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Assessing the Risk of Histamine from the Indonesian Salted-Boiled Fish (*pindang*)

By

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This thesis comprises of work that has been prepared to be submitted to journals. Information for each chapter is provided in the section of communications arising from this thesis.

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Authors: Novalia Rachmawati, Shane M. Powell, Mark L. Tamplin, and Tom Ross.

Author 1 contributed 70% (designed the experiment, conducted the field work and laboratory analysis, analysed the data and wrote the manuscript), author 2, 3 and 4 each contributed 10% (provided suggestion for the experimental design, contributed to the molecular ecology data analysis and provided input for the manuscript).

Paper 2. Modelling the growth of *Enterobacter aerogenes* in conditions that mimic the processing of the Indonesian Salted-Boiled Fish

Journal article in preparation

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List of Abbreviations

AOAC	Association of Official Analytical Chemists
API	Analytical Profile Index
ARISA	Automated Ribosomal Intergenic Spacer Analysis
CCPs	Critical Control Points
CFU	Colony Forming Units
CTAB	Cetyl trimethylammonium bromide
ELISA	Enzyme-Linked Immunosorbent Assay
HACCP	Hazard Analysis and Critical Control Points
Hdc	Histidine decarboxylase (enzyme)
hdc	histidine decarboxylase (gene)
HFP	Histamine Fish Poisoning
HPB	Histamine Producing Bacteria
ITS	Integral Transcriber Spacer
MRA	Microbiological Risk Assessment
NCBI	The National Center for Biotechnology Information
RMSE	Root-Mean-Square Error
SNI	Standar Nasional Indonesia (The Indonesian National Standard)
SPE	Solid Phase Extraction
UPLC-MS	Ultra-Performance Liquid Chromatography – Mass Spectrometry
w/v	weight/volume
w/w	weight/weight

Abstract

Indonesian salted-boiled fish, known as *pindang*, is the second largest traditional fish product in Indonesia and has important economic and social impacts for Indonesian societies, especially those who live in coastal areas. *Pindang* is made from Scombroid fish, such as tuna, mackerel and scad. *Pindang* processors are family- or neighbourhood-based industries equipped with basic or traditional processing equipment, with processing techniques passed from generation to generation. Salting and steaming (or boiling) are combined as methods of preservation in *pindang* processing. Some of the largest *pindang* processing centres are located in Pelabuhan Ratu-Sukabumi District (West Java Province), Juwana (Central Java Province) and Klungkung (Bali Province).

As with many other Scombroid-based products, *pindang* has a high risk of being contaminated with histamine. Reports indicate that *pindang* has caused several histamine fish poisoning (HFP) outbreaks in Indonesia. HFP is one of the major problems in seafood industries worldwide. This foodborne intoxication is caused by the ingestion of fish or other foods containing high levels of histamine. The formation of histamine depends on the availability of free histidine in fish flesh and the presence of histamine-producing bacteria (HPB) that harbour the histidine decarboxylase (Hdc) enzyme and convert histidine into histamine. Histamine is a heat-stable amine and cannot be destroyed by common cooking practices such as boiling, steaming, heating or freezing.

In Pelabuhan Ratu, Sukabumi District, West Java Province, Indonesia, *pindang* processing relies on the availability of fresh tuna from the local catchment. However, due to the limited number of fresh fish caught from the surrounding waters, *pindang* processors use frozen tuna as an alternative raw material. When frozen fish is used, a thawing step is introduced in the preparation phase of *pindang* processing. Improper thawing practices of raw material may lead to HPB growth and histamine formation. Therefore, this study aimed to provide a better understanding of the

current processing of *pindang* in Pelabuhan Ratu and how the processing affects histamine accumulation in the final product.

Field observations and laboratory experiments were combined to collect relevant data on *pindang* processing, to evaluate the behaviour of HPB isolated from *pindang* and to identify intervention strategies which can be applied to prevent histamine accumulation in the final product as well as to improve the safety and quality of the product.

To identify HPB that are commonly found in *pindang,* fish samples were collected from several *pindang* processors in Pelabuhan Ratu. Fish from different processing stages (raw, thawed/washed, and cooked) were sampled. HPB were screened using modified Niven's agar and a PCR-based assay and identified using the API system and 16S rRNA gene sequencing. The ability of the isolates to produce histamine was evaluated using histidine decarboxylase broth and an ELISA method. In total, fourteen different HPB genera were identified, *i.e*. *Citrobacter, Enterobacter, Escherichia, Erwinia, Hafnia, Klebsiella*, *Morganella, Pantoea, Proteus, Providencia, Pseudomonas, Serratia, Shigella* and *Vibrio*. In particular, *Enterobacter* sp., *Klebsiella* sp., *Morganella morganii* and *Providencia* were found on thawed fish. Further confirmation showed that an *Enterobacter aerogenes* isolate produced ≥ 4,000 µg/ml histamine in histidine-rich broth media after 24 h incubation at 30°C.

A predictive modelling study was done to understand the growth of *E. aerogenes* isolated from *pindang* and its ability to produce histamine, at different conditions relevant to *pindang* production. The isolate was inoculated into histidine broth with different concentrations of salt (1.5, 6, 10 and 20% w/v) and in fish with 6% of salt concentration, then incubated at 10, 15, 20 and 30°C. Growth and histamine formation during the incubation were recorded. The growth parameters (growth rate-μ and lag time-λ) of *E. aerogenes* were modelled using the Roberts and Baranyi model as implemented in the DMFit version 3.5 Excel® add-in. A multiple linear regression and the extended Ratkowsky model were used to describe the effect of temperature and salt on the growth

of *E. aerogenes*. Histamine formation during incubation was determined by combining a yield factor to the growth model. The results showed linear relationships among the bacterial growth rate, with temperature and salt. In broth experiment, the highest histamine level (>6,000 µg/ml) was produced at 30°C with 1.5% of salt. Although the isolate survived at 10% salt, the amount of histamine produced was very low (less than 10 µg/ml).

To evaluate the growth and histamine formation of *E. aerogenes* under conditions that mimic the thawing process of *pindang*, a broth experiment was done by growing the isolate to different cell densities and freezing at -20°C for 72 h. Thawing of the frozen culture was done at 4, 18 and 25°C for 4 h. The bacterial growth and histamine formation during the thawing process were recorded. The inoculum was reduced from the initial counts after freezing, and no bacterial growth was observed during subsequent thawing at different temperatures for 4 h. For treatment with high initial bacterial counts, the histamine production during thawing was more pronounced than treatment with low initial counts.

To describe the existing processing practices of *pindang,* field observations were done at several processors located in Pelabuhan Ratu. Two types of *pindang* processing were observed. The first processing type used fresh Skipjack tuna, while the second used frozen Little Eastern tuna. *Pindang* processing took four to five hours, depending on the type of raw materials used. When frozen fish were used as raw material, the processing required an additional 60 - 90 minutes of preparation, which predominantly was thawing. The histamine levels of fresh Skipjack and *pindang* made from this fish were very low (less than 50 µg/g). However, when frozen Little tuna was used as raw material, the histamine levels of some raw fish were higher than 100 µg/g. This greater level of histamine was also observed for cooked fish. Microbial community profiles for fish from different sources and at different processing steps, identified using the Automated Ribosomal Intergenic Spacer Analysis (ARISA), showed that the bacterial composition of *pindang* was affected by the type of fish used as raw material, fish processing (salt addition and heating), as well as post-processing

contamination. Six critical control points (CCPs) were identified from *pindang* processing, *i.e.* receiving raw materials, thawing and washing, paper wrapping and arranging fish in the cooking container, salting, cooking and post-process handling.

The first CCP in *pindang* processing is the choice of raw materials. To prevent high histamine levels in the final product, the histamine levels of raw fish should not exceed the allowable limit of fresh fish (100 mg/kg as regulated by the Indonesian Standardization Body). For processing units that use frozen fish as raw material, the second CCP is the thawing and washing step. Thawing becomes a critical step as temperature abuse and time delays likely occur during this step. These conditions allow HPB, in particular, to grow and to produce histamine. Since histamine is a heat-stable amine, the subsequent processing steps of *pindang*, *e.g.,* paper wrapping, salting and cooking, do not eliminate pre-formed histamine.

In general, the histamine levels of raw tuna used in *pindang* processing will determine the histamine levels in the final product. When fresh tuna is not available, frozen tuna can be used as raw material. However, the temperature during the preparation step, especially during thawing and washing should be maintained below 18°C to avoid bacterial activity and histamine formation. Furthermore, the use of higher concentrations of salt (10%) is recommended to prevent the growth of halophilic HPB that may otherwise survive salting. Improved hygienic practices during processing are also necessary. For example, clean and circulated water should be used during thawing and washing to prevent contamination from the environment. Proper packaging combined with low storage temperature of the cooked product might also prevent post-processing contamination and increase the product shelf-life.

Chapter 1. Introduction and Literature Review

1.1. Introduction

Histamine fish poisoning (HFP) is a disease caused by the ingestion of fish or other foods containing high levels of histamine. HFP is one of the major problems in seafood safety worldwide. The combination of temperature abuse and mishandling of fish during processing, storage and distribution have been identified as the main cause of histamine formation and accumulation in fish products (Baylis, 2006; FAO & WHO, 2012; Hungerford, 2010; Taylor, 1986; Taylor et al., 1989).

The first part of this literature review provides a general introduction to fish and fish product safety followed by a summary of HFP and factors that contribute to the formation and accumulation of histamine in fish products. Different aspects of histamine including the metabolism pathways, toxicity and detection methods are further discussed. A short introduction on Indonesian *pindang*, as the main subject of this research, and the present problems of HFP related to this product are also presented.

The second part of the literature review describes microbiological risk assessment and emphasises the application of predictive modelling and quantitative microbiological risk assessment as a powerful tool to assess and improve the microbiological safety of foods. Different strategies to control histamine formation and accumulation during the processing of the Indonesian *pindang* are also discussed. Finally, the objectives of the research will be outlined.

1.2. Fish production, utilisation and safety

Fish is an important constituent of the human diet. It contains protein and lipids as the major components and essential micronutrients such as vitamins (A, B and D), minerals (calcium, iodine, zinc, iron and selenium), and polyunsaturated fatty acids (FAO, 2016).

According to a report from the Food and Agriculture Organization (FAO), global fish production reached 167.2 million tonnes in 2014, with 93.4 million tonnes from wild capture and

73.8 million tonnes from aquaculture fisheries (FAO, 2016). The biggest producer of both capture and aquaculture fisheries in 2014 was China, followed by Indonesia as the second and third largest global contributor for capture and aquaculture fisheries, respectively. Indonesia produced 6.4 million tonnes of total inland and marine waters capture and 4.2 million tonnes of aquaculture fishery products in 2014 (FAO, 2016).

Fish consumption by the Indonesian population per capita increased from 33.89 kg in 2012 to 43.88 kg in 2016 (MMAF Center of Data Statistic and Information, 2017). These values are much higher than the global fish consumption which was predicted to be above 20 kg per capita in 2013/2014 (FAO, 2016). Moreover, fish contributed 17% of animal protein consumed by the global population in 2013, whereas in some countries such as Indonesia, Bangladesh, Cambodia, Ghana, Sierra Leone and Sri Lanka, it contributed 50% or more of the total animal protein intake (FAO, 2016).

From the total fish production globally, more than 146 million tonnes of fish were utilized for human consumption in the forms of live, fresh and chilled fish (46%), while the rest was processed into dried, salted, smoked and other preserved fish (12%), prepared and preserved fish (13%), and frozen fish (30%) (FAO, 2016). In Indonesia, the production of processed fish reached 1.2 million tonnes in 2012 with traditional processed fish (dried/salted fish, smoked, boiled, fermented) composing 57% of the total production, followed by frozen and canned fish that composed 39% and 0.6% of the total production, respectively (MMAF Center of Data Statistic and Information, 2012). Amongst other traditionally processed fish, *pindang* has been chosen in 2012 as one of the products targeted for industrialisation based on the Ministerial Regulation PER.27/MEN/2012 issued by the Indonesian Minister of Marine Affairs and Fisheries on the general guidelines of marine and fisheries industrialisation in Indonesia.

1.2.1. Histamine Fish Poisoning (HFP)

Bacterial foodborne diseases are categorised into infection and intoxication. The infection mainly occurs due to ingestion of fish or foods containing live bacteria that will continue to grow in human body and will cause the symptoms of infection, while intoxication occurs when a person consumes toxins that are already formed in fish or food products as a result of bacterial metabolic process (Huss & Gram, 2004), such as biogenic amines. HFP was formerly termed 'scombroid fish poisoning' since the cases usually correspond with scombroid fish that have high levels of free histidine such as tuna and albacore (*Thunnus*), skipjack (*Katsuwonus*), mackerel (*Scomber*) as well as bonitos (Taylor, 1986). However, other types of fish were also sources of HFP, including bluefish (*Pomatomus*), bluefin trevally (*Caranx*), redfish (*Sebastes*), herring (*Clupea*), mahi-mahi (*Coryphaena*), sardines and anchovies (Kasuga, 2005; Lehane & Olley, 2000). Some species with low levels of free histidine, such as Western Australian salmon with histidine level ranging from 20 to 30 ppm, have also been associated with the cases of HFP (Bartholomew et al., 1987; Smart, 1992; Wilson & Cowey, 1985). An example of fish and fish products implicated in HFP cases in the period of 2000 – 2016 and the level of histamine measured in the fish, is presented in Table 1-1 while the confirmed reports and notifications of HFP due to the consumption of fish and fish product based on the US CDC National Outbreak Reporting System (NORS) and the EU Rapid Alert System for Food and Feed (RASFF) are presented in Table 1-2 and Table 1-3, respectively. There were 197 confirmed and 16 suspected reports of illnesses from CDC (https://wwwn.cdc.gov/norsdashboard/), while 73 notifications were received from the EU member states in the RASFF during that period (https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList).

Table 1-1. Fish and fish products implicated in HFP cases reported during 2000 - 2016

Fish name	Histamine level (mg/kg)	Reference
Escolar (*Lepidocybium flavobrunneum*)		
Cooked, marinated, smoked	400 - 7,300	Emborg et al. (2008); Feldman et al. (2005); Kan et al. (2000)
Fish cube (*Tetrapturus angustirostris*)	523	Chen et al. (2010a)
Marlin fillet (*Makaira nigricans*)	435 - 478	Chen et al. (2010b)
Canned mackerel	612 - 1,539	Tsai et al. (2005b)
Milkfish (*Chanos chanos*)	616 - 2,350	Lee et al. (2016); Tsai et al. (2007)
Mahi-mahi (*Coryphaena hippurus*)	113 - 2,000	Adams and Langley (2004); Chen et al. (2011); McLaughlin and Gessner (2008)
Saury paste (*Cololabis* sp.)	320	Miki et al. (2005)
Swordfish (*Xiphias gladius*)		
Filleted, fried, cooked	280 - 7,600	Chang et al. (2008); Emborg et al. (2008); Otani et al. (2004)
Frozen	6,700	Otani et al. (2004)
Sardine		
Canned, dried	144.8 – 1,700	Kanki et al. (2004); Petrovic et al. (2016)
Tuna		
Cold-smoked	914 - 4,550	Emborg et al. (2008)
Fresh, raw	96 - 1,738	Emborg et al. (2008)
Processed or prepared products (burger, canned, dumpling, fermented, fried, mayonaise, patties. ready to heat packed, salad, sandwich)	86 - 6,432	Adams and Langley (2004); Becker et al. (2001); Chen et al. (2008); Emborg et al. (2008); Hongchumpon et al. (2016); McCormick et al. (2013); Predy et al. (2003)
Yellowfin tuna (*Thunnus albacares*)	470 – 4,900	Demoncheaux et al. (2011); Ohnuma et al. (2001)
Vacuum packed	7,100 - 9,100	Emborg et al. (2005)

Table 1-2. Reported illnesses due to histamine in the US during 2000 – 2016

Fish name	Number of confirmed reported outbreaks and year occurred
Ahi	14 (2001), 3 (2002), 2 (2004), 13 (2009)
Blue marlin	6 (2001), 6 (2003)
Escolar	27 (2001), 42 (2003), 3 (2004), 8 (2005), 5 (2006)
Mahi-mahi	2 (2000), 4 (2002), 8 (2003), 3 (2008)
Marlin	5 (2012)
Tuna	16 (2000), 2 (2004), 6 (2006), 2 (2011), 9 (2012), 3 (2013)
Wahoo	2 (2010)
Yellowfin tuna	4 (2007), 2 (2012)

Table 1-3. Histamine food poisoning notifications due to the consumption of fish and fish products in the EU during 2000 – 2016

Fish name	Histamine level (mg/kg)	Year	Number and type of notification
Anchovies (*Engraulis encrasicolus*) Fillets, in oil	290; >1,920; 4,200	2013	1 alert, 1 information for attention
Butterfish/Escolar (*L. flavobrunneum*)	<5 - 208; <50 - 1,000	2009 and 2012	1 alert, 1 information
Marlin	400	2016	1 alert
Sardines	220.3 - 1,323	2013	1 information, 1 information for attention
Yellowfin tuna (*T. albacares*) Chilled, vacuum packed, fresh, frozen, processed, raw, defrozen	1 - 5,100	2008-2016	9 alert, 9 information for attention, 7 information, 4 suspected cases
Albacore (*Thunnus alalunga*)	3110; 3,251-3,698	2010 and 2014	1 alert, 1 information for attention
Atlantic Bluefin Tuna (*Thunnus thynnus*)	>1,000; 3,603	2009 and 2010	1 alert, 1 information
Tuna Canned, chilled, defrosted, fresh, frozen, raw, thawed, vacuum packed	5 - 10,000	2008 - 2016	10 alert, 6 information, 13 information for attention, 3 suspected cases
Yellowtail amberjack (*Seriola lalandi*)	1,000	2009	1 information

Since the HFP cases were not only caused by Scombroid fish but also different types of fish, the term 'histamine fish poisoning' is now considered more relevant than 'scombroid fish poisoning'.

In Indonesia, *pindang* is known as one of the major causes of HFP and outbreaks have occurred in different provinces. A pilot project of Indonesian RASFF (INRASFF) was initiated by the National Agency of Drug and Food Control (NA-DFC) of Indonesia in 2010 but is still underway, thus the number of foodborne outbreaks, including those caused by HFP are difficult to estimate using the INRASFF platform. However, in the last few years, several HFP incidents have been recorded including an outbreak in Sleman, Yogyakarta Province, where up to 166 workers from a factory suffered HFP after consuming *pindang* prepared by a caterer; 19 of them received intensive treatment in local hospitals (Anonymous, 2004). In Sukawati, Bali, 40 workers reported suffering from HFP after consuming *pindang* from a local market; seven of them received intensive treatment (Anonymous, 2008). In West Java, there were at least two outbreaks of HFP due to the consumption of *pindang* recorded from different locations, Sukabumi and Tasikmalaya, in 2014 and 2016, respectively, during which more than ten people experienced dizziness, vomiting and some were hospitalised (Anonymous, 2014, 2016). In 2015, a similar outbreak occurred where 34 Chief of Districts suffered from HFP after eating *pindang* in Malang, East Java (Ainun, 2015).

1.2.2. HFP clinical symptoms

HFP symptoms usually occur within a few minutes to a few hours after consumption of affected product and may persist for 12 hours to a few days (US DHHS, 2011). The symptoms are similar to histamine intolerance or an allergic reaction, except that during clinical cases of HFP the immune system is not involved in the reaction, and it occurs not only to persons susceptible to histamine but also to non-susceptible persons (FAO & WHO, 2012; Hungerford, 2010). There are four receptors of histamine located in the different part of the body and targeted cells in various tissue, *i.e.* H1R, H2R, H3R and H4R. These receptors trigger different reactions such as muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion, tachycardia,

alteration of blood pressure, and stimulation of gastric acid secretion (Maintz & Novak, 2007). The clinical manifestations of these reactions are observed in skin, the gastrointestinal tract, and the nervous, cardiovascular, and respiratory systems; the severity of the symptoms may vary between individuals, depending on their medical history (FAO & WHO, 2012; Maintz & Novak, 2007; Taylor et al., 1989; Tortorella et al., 2014). Several reported symptoms of HFP are summarised in Table 1-4. Due to the mildness of the symptoms, HFP is usually self-medicated and most people recover within 24 hours, thus many cases remain unreported (Hungerford, 2010; Knope et al., 2014; Tortorella et al., 2014).

Table 1-4. Clinical symptoms of reported HFP cases

Clinical symptoms	Reference
A peppery taste, numbness of the tongue, headache, flushing and sweating, dizziness, nausea, diarrhoea, and dyspnea	Feldman et al. (2005)
Facial flushing and pruritic rashes on the face, neck, and trunk, heart palpitations, diarrhoea, headache, abdominal cramping	Davis et al. (2007)
Rash, flushing, headache, erythema, tachycardia, diarrhoea, dyspnea, abdominal pain, pruritus	Lavon et al. (2008)
Tongue and face swelling, rashes, headache, dyspnea	Wilson et al. (2012)
Skin rashes, mouth tingling, vomiting, dyspnea (difficulty breathing)	McCormick et al. (2013)
Vomiting, headache, skin rashes, hives	Anonymous (2014)
Diarrhoea, rash, nausea, vomiting, fever, abdominal pain	Knope et al. (2014)
Palpitations, headache, hypotension, erythema, eyelid oedema	Tortorella et al. (2014)
Nausea, vomiting, headache, skin rashes	Ainun (2015)

1.3. Histamine toxicity in human and the role of potentiators

In the human body, free histidine is also metabolised through the catabolic reaction of L-histidine ammonia lysase (HAL), producing urocanic acid, glutamate and α-ketoglutarate, that enter the citric acid cycle, and the decarboxylation of histidine by the Hdc enzyme to produce histamine (Lehane & Olley, 2000).

Ingestion of histamine from a spoiled fish caused more severe toxic reactions compared to pure histamine taken orally which is suggested to demonstrate the role of potentiators in histamine toxicity (Ababouch & Gram, 2004; Lehane & Olley, 2000; Taylor, 1986). Cadaverine and putrescine have been suggested as histamine potentiators. Although oral administration of histamine together with these potentiators increased the toxicity of histamine in test animals (Taylor, 1986), the roles of these biogenic amines in HFP remain debated.

Several mechanisms of histamine potentiators have been proposed, *i.e.* the inhibition of diamine oxidase (DAO) and histamine N-methyltransferase (HNMT), the disruption of intestinal mucosa as a protective layer and the production of endogenous histamine from mast cells (Ababouch & Gram, 2003; Prester, 2011). DAO and HNMT are the major enzymes that detoxify histamine and are found in the human intestinal tract and liver. The inhibition of these enzymes by the potentiators can increase histamine absorption in the gut and prevent histamine metabolism in extra-intestinal tissues. The second mechanism suggests that potentiators are able to disrupt the barrier function of the small intestine, and thus increase histamine toxicity by increasing histamine transport across the gut. The third mechanism proposes that the production of a cell mass degranulator releases endogenous histamine from histamine-heparin complex mass cells. However, the significance of mass degranulator in the toxicity of histamine remains unproven (Hui & Taylor, 1985; Hungerford, 2010; Lehane & Olley, 2000).

Furthermore, the formation of biogenic amines in fish is species specific (Prester, 2011), which adds to the complexity in determining the role of potentiators in histamine toxicity. Although commonly found in the decomposed fish, the levels of cadaverine and putrescine are considerably lower than histamine. Other biogenic amines including tyramine, spermine, spermidine, dopamine and agmatine, are also found in fish but in lower amounts and do not clearly contribute to HFP cases (FAO & WHO, 2012; Visciano et al., 2012).

1.4. Histidine metabolism in fish

Biogenic amines are naturally present in fruits and vegetables, and also in other types of food including fish, fish products, meat products, eggs, and fermented products such as cheeses, beers, wines, fermented soybean and fermented vegetables. They are formed through decarboxylation of amino acids by bacteria that are present during food handling and storage (Shalaby, 1996). Histidine is the precursor of histamine and appears to be the main factor in histamine formation (Borgstrom, 1961).

Histidine is metabolised through two different pathways, *i.e.* catabolism by histidase to form glutamic acid, which is converted to alpha-ketoglutarate, an intermediate in the Krebs cycle, or by decarboxylation by the enzyme histidine decarboxylase (Hdc) to form histamine (Tortorella et al., 2014). Decarboxylation of histidine in fish is mediated either by the decarboxylase enzyme produced endogenously in fish muscle or by a similar enzyme produced exogenously by bacteria present in the fish (Lehane & Olley, 2000; Rawles et al., 1996). However, under normal storage conditions for histamine-rich foods, histamine formation due to bacterial decarboxylation is more pronounced than fish muscle decarboxylation (Fernandez-Salguero & Mackie, 1979; Landete et al., 2008; Lehane & Olley, 2000).

Hdc is generally produced by bacteria belonging to the *Enterobacteriaceae* group, although other groups of bacteria are also able to produce the enzyme (FAO & WHO, 2012; Kimura et al., 2009; Lehane & Olley, 2000; Takahashi et al., 2003; Visciano et al., 2012). Two different bacterial Hdc enzymes have been intensively studied, *i.e.* pyridoxal phosphate- and pyruvoyl-dependent Hdc, which are found in Gram-negative and Gram-positive bacteria, mainly lactic acid bacteria, respectively (Landete et al., 2007, 2008). Both enzymes have similar Hdc activity, although the molecular organisation and substrate specificity are different. The pyridoxal phosphate-dependent Hdc has an optimum pH of 6.8 and is relatively unstable, while pyruvoyl-dependent Hdc is more stable and most active at pH 4.8 (Landete et al., 2008).

The nucleotide sequences of the *hdc* genes have been studied from different Gram-negative and Gram-positive bacteria. In general, *hdc* from Gram-negative bacteria consists of 1) putative histidine/histamine antiporter (*hdcT*) that transports histidine inside the cell and transports histamine out of the cell, 2) *hdc* which catalyses the conversion of histidine into histamine and, 3) putative histidyl-tRNA synthetases (*hisRS* or *hisS*) that play a role in gene regulation (Bjornsdottir-Butler et al., 2016). Kamath et al. (1991) found the pyridoxal-dependent *hdc* from *Klebsiella planticola* and *Enterobacter aerogenes* have 85 and 80% sequence homology with *Morganella morganii* AM-15, in which the latter has two *hdcT* (Vaaler et al., 1986) while *K. planticola* and *E. aerogenes* have an additional *hisRS2*. Further study showed that *M. morganii* DSM30146 has four open reading frames, *hdcT1, hdc, hdcT2* and *hisRS* (Ferrario et al., 2014). *Photobacerium damselae* subsp. *damselae* and *Photobacterium angustum* each have three ORFs, *hdcT*, *hdc* and *hisRS* (Bjornsdottir-Butler et al., 2016; Kimura et al., 2009), and *Photobacterium kishitanii* has three ORFs, *hdcT*, *hdc* and *hisRS* (Bjornsdottir-Butler et al., 2016). In contrast, *hdc* of Gram-positive bacteria usually consists of the pyruvoyl-dependent encoding gene (*hdcA*), and histidine/histamine antiporter (*hdcP*) (Rossi et al., 2011). In addition, the *hdcB* gene was recently found to encode a functional enzyme that catalysed maturation of the pyruvoyl -dependent *hdcA* in *Streptococcus thermophilus* (Trip et al., 2011). Other species of lactic acid bacteria such as *Oenococcus oeni*, *Lactobacillus* sp. and *Tetragenococcus* sp. also showed similar clusters of *hdc* that consist of a transporter (*hdcP*), the encoding genes (*hdcA*) and *hdcB* gene (Calles-Enríquez et al., 2010).

Available information on gene sequences is essential to develop molecular techniques that may enable rapid detection and identification of HPB. Furthermore, this information can be used to study the activities of *hdc* at different conditions, including those relevant to food production (Bjornsdottir-Butler et al., 2016; Rossi et al., 2011; Tabanelli et al., 2012).

1.5. Histamine producing bacteria (HPB)

The composition of macro and micronutrients of fish creates a suitable environment for bacteria to grow. The high proportion of non-protein nitrogen (NPN), such as free amino acids and ammonia, in fish muscle provide favourable substrates for bacteria during the decomposition phase, and result in fish being a more perishable product than other animal products. Although the internal parts of fish muscle are generally sterile, the natural microflora in the surrounding organs such as gills, intestine and skin, and the fish environment, can potentially transfer bacteria into the fish muscle during post-harvesting stages (Kasuga, 2005; Mayer & Ward, 1991). Amongst the natural fish microflora, HPB comprise about 1% of total diversity of the surface microflora in live fish, while the remaining are non-histamine forming species and are most likely found in fish gills, skin or the gastrointestinal tract (FAO & WHO, 2012; Kimata, 1961; Lehane & Olley, 2000). This group of bacteria plays an important role in HFP. Early fish evisceration and gill removal after catch/capture have been suggested as a means to eliminate the presence of HPB, however improper processing techniques may cause the spread of HPB into the fish flesh and initiate the formation of histamine (Visciano et al., 2012).

Enterobacteriaceae species such as *M. morganii*, *Raoultella planticola*, *Raoultella ornithinolytica, Hafnia alvei*, *Klebsiella pneumonia*, *Citrobacter freundii, E. aerogenes* and *Proteus vulgaris* are usually implicated in HFP cases related to the consumption of fish and fish products (Björnsdóttir-Butler et al., 2010; Emborg & Dalgaard, 2006; Kanki et al., 2004; Lehane & Olley, 2000; Visciano et al., 2012). Other genera of HPB have also been isolated from spoiled fish, such as *Clostridium perfringens* from decomposing skipjack tuna (*Katsuwonus pelamis*) (Yoshinaga & Frank, 1982) and *Pseudomonas* from spoiled marine tropical seafood stored in ice (Gram & Huss, 1996; Koutsoumanis & Nychas, 1999). Most of these named species are mesophilic bacteria that optimally grow at 30-40°C and are able to convert histidine into high levels of histamine (Table 1-5).

HFP cases have been previously associated only with mesophilic bacteria. However, several recent studies showed that HFP is also associated with psychrotolerant bacteria, such as *M. psychrotolerans* and *P. phosphoreum* which is found in fish stored at refrigerated or frozen temperatures (Emborg & Dalgaard, 2008b; Hungerford, 2010; Torido et al., 2014). A histamine poisoning case associated with sashimi, tuna and marlin fillet occurred in Japan during the cold season in 2002, and based on microbial examination, *P. phosphoreum* was suspected as the main causative agent in frozen-thawed fish (Kanki et al., 2007). *P. phosphoreum* are natural microflora of temperate water fish (Torido et al., 2014; Visciano et al., 2012). Emborg and Dalgaard (2008a) and Emborg and Dalgaard (2008b) categorised *P. phosphoreum* as psychrotolerant HPB which are able to grow at low temperature and have an optimal growth temperature of 25°C (Morii et al., 1994), while Kanki et al. (2004) classified this bacteria as psychrotropic HPB which produced significant level of histamine at 12 and 20°C. *P. phosphoreum* was not previously isolated and identified from products associated with HFP since this isolate is not able to grow at high temperature (37°C) (Emborg, 2007), and it requires higher salt concentration in the media as for marine bacterial examination (Kanki et al., 2007). Thus this bacteria was not identified using the common agar identification and incubation methods of HPB. However, a recent study by Torido et al. (2014) found that psychrotolerant bacteria can be isolated from a wide range of fish, although fewer species produce more than 1,000 mg/kg of histamine, compared to mesophilic bacteria. Therefore, assessment of fish products implicated in HFP cases should also consider the psychrotolerant HPB.

Table 1-5. Mesophilic HPB isolated from different fish products

Species	Histamine level (mg/kg)	Fish sources	Reference
Morganella morganii	2,600 - 3,870	Sardine (*Sardina pilchardus*)	Ababouch et al. (1991)
	59	Fried fish fillet implicated in HFP	Lee et al. (2012)
	> 1,000	Bonito, tuna, yellowtail, flying fish, mackerel, saury, sardine, swordfish	Torido et al. (2014)
	900 – 1,460	Mackerel, albacore, mahi-mahi and tuna stored at 37°C	Kim et al. (2002)
Raoultella planticola	> 1,000	Tuna	Torido et al. (2014)
Raoultella ornithinolytica	562	Fried fish fillet implicated in HFP	Lee et al. (2012)
	561.7	Dried mahi-mahi	Lin et al. (2014)
	> 1,000	Tuna	Torido et al. (2014)
Hafnia alvei	108 - 153	Fresh albacore tuna	Kim (2001)
	2,002	Mackerel stored at 25°C	Jiang et al. (2013)
Klebsiella pneumonia	4,420	Tuna sashimi implicated in HFP	Taylor et al. (1979)
Citrobacter freundii	> 200	Swordfish, tuna	Torido et al. (2014)
Enterobacter aerogenes	287	Fried fish fillet implicated in HFP	Lee et al. (2012)
	> 1,000	Tuna, mackerel	Torido et al. (2014)
Proteus vulgaris	156	Mahi-mahi fillet	Lin et al. (2014)
	125 - 728	Mackerel stored at 25°C	Jiang et al. (2013)

Gram-negative HPB are commonly associated with fresh or raw fish, while histamine in fermented fish is mostly produced by Gram-positive bacteria (Landete et al., 2006). Chang et al. (2008) isolated *Staphylococcus* spp. and *S. aureus* from swordfish fillets containing 859 - 2,937 ppm histamine, associated with HFP in Taiwan. When grown in histidine supplemented media, the isolates produced 12 – 33 ppm histamine. *Bacillus* spp., another Gram-positive bacterial genus, have been isolated from various fermented fish products sold in Taiwan supermarkets. Although able to produce histamine at 8 – 13 ppm, *Bacillus* spp. are not the main contributors of histamine accumulation in the products (Tsai et al., 2006). Control of biogenic amines in fermented products is more achievable, for example with the addition of a starter culture with low Hdc activity, or the use of cultures containing amine oxidase to degrade histamine (Chong et al., 2011).

1.5.1. Identification of HPB

A differential agar, consisting of 0.5% tryptone, 0.5% yeast extract, 2.7% L-histidine.2HCl, 0.5% NaCl, 0.1% CaCO3, 2.0% agar, and 0.006% bromocresol purple, with pH adjusted at 5.3, was introduced by Niven et al. (1981) for qualitative identification of HPB. Decarboxylase activity is recognised by the media colour change (Kim et al., 2001; Shelef et al., 1998). As bacteria metabolise glucose into acid the media changes from purple to yellow. In addition, the metabolism of amino acid into amines increases the media pH and reverses the media colour into purple. Therefore, purple colonies indicate the presumptive HPB, while yellow colonies represent the non-HPB. Despite the popularity of Niven's media as a simple approach to identify HPB, several studies have identified drawbacks to this method, including false-positive results due other metabolic processes that also increase pH, loss of histamine production of culture-based isolates, and false-negatives due to low medium pH that inhibit bacterial growth (Allen et al., 2005; Björnsdóttir-Butler et al., 2010; Kim et al., 2001). Modification of Niven's medium has been proposed by changing pH, incubation time and temperature (Kim, 2001; Mavromatis & Quantick, 2002), and pre-screening on selective agar, which increases detection of HPB (Yoshinaga & Frank, 1982). However, this media is still widely used as a

screening tool of presumptive HPB, including identification of low histamine producing isolates, because it is easy, inexpensive and reliable (Bjornsdottir et al., 2009).

Tuna fish infusion broth (TFIB) and trypticase soy broth fortified with histidine (TSBH) were also proposed as media for identification and enumeration of HPB. TFIB is prepared from raw tuna homogenate in water at a ratio 1:2 (w/v) (Omura et al., 1978). This homogenate is steamed at 100°C for 1 hr, filtered, then enriched with 1% glucose and sterilised before use. TSBH is made by supplementing TSB medium with 0.1% of histidine (Taylor & Woychik, 1982). The composition of TFIB represents more favourable conditions for HPB to grow than TSBH, including high conversion rate of histidine into histamine in TFIB. However, the exact composition of TFIB is unknown and depends on the quality of raw tuna used in the preparation (Taylor & Woychik, 1982).

A rapid and reliable method of HPB identification is important to detect their presence in fish and avoid histamine accumulation in the later processing stages. Molecular methods targeting *hdc* genes offer more precise identification of HPB. Le Jeune et al. (1995) and Alves et al. (2002) developed PCR methods to detect pyruvoyl-dependent *hdc* encoding genes of Gram-positive bacteria; different sets of primers for the detection of Gram-negative bacteria are summarised in Table 1-6.

Table 1-6. Primers used in the identification of Gram-negative HPB

Primer name	Sequence (5'---3')	Amplicon size (bp)	Note	Reference
KPF2	AAA GCT GGG GGT ATG TGA CC	724	Based on *hdc* genes of *R. planticola*	Kanki et al. (2002)
KPR4	GTG ATG GAG TTT TTG TTG C			
Mm208F	CTC GCA CCA TCA GAT GAA CCC ATA T	809	Based on *hdc* genes of *M. morganii*	Kim et al. (2003b)
Mm1017R	CAA AGC ATC TCT GCT AAG TTC TCT GGA TG			
hdc-f	TCH ATY ARY AAC TGY GGT GAC TGG RG	709	Based on *hdc* genes of *M. morganii, Enterobacter aerogenes,* and *R. planticola*	Takahashi et al. (2003)
hdc-r	CCC ACA KCA TBA RWG GDG TRT GRC C			
106	AAYTCNTTYGAYTTYGARAARGARG	534	Based on *hdc* genes of *M. morganii, R. planticola* and *Pseudomonas fluorescens*	de Las Rivas et al. (2005)
107	ATNGGNGANCCDATCATYTTRTGNCC			
HIS1-F	GGNATNGTNWSNTAYGAYMGNGCNGA	372	Based on *hdc* genes of *Lactobacillus* sp. 30a (ATCC 33222) and *M. morganii* CECT 173^{T} (ATCC 33539)	
HIS1-R	ATNGCDATNGCNSWCCANACNCCRTA			
HIS2-F	AAYTSNTTYGAYTTYGARAARGARGT	531	Based on *hdc* genes of *P. damselae* CECT 626^{T} (ATCC 33539), *P. phosphoreum* CECT 4192^{T} (ATCC 11040), and *P. vulgaris* CECT 484^{T} (ATCC 13315)	(de las Rivas et al., 2006)
HIS2-R	TANGGNSANCCDATCATYTTRTGNCC			

Instead of identifying a single HPB, some of those studies developed an assay to detect multiple biogenic amine producing bacteria that can be routinely used for early detection (de las Rivas et al., 2006; de Las Rivas et al., 2005).

Colony lift hybridisation using a specific probe for the *hdc* gene and real-time PCR assays have also been developed to identify HPB from fish samples. These methods have high specificity for HPB that produce high levels of histamine, but cannot identify or distinguish between low and non-HPB

(Björnsdóttir-Butler et al., 2010; Bjornsdottir-Butler et al., 2011a). This drawback is mainly due to unavailability of information on the *hdc* sequences of low histamine producers (Björnsdóttir-Butler et al., 2010).

Despite fast and reliable results, most of the PCR methods still fail to recognise low histamine producers and require additional steps to confirm the isolate (Björnsdóttir-Butler et al., 2010). More advanced techniques such as a combination of a culture based-method and nucleic acid hybridization and species-specific protein markers have recently been developed to provide high specific species fingerprint that can be used as an identification tool of unknown HPB, including the low histamine producers (Björnsdóttir-Butler et al., 2010; Böhme et al., 2010; Fernandez-No et al., 2011; Fernandez-No et al., 2010).

1.6. Histamine formation during fish production

Several studies in broth and fish products recorded that histamine formation correlates to the HPB growth phase (Table 1-7), especially for mesophilic HPB which were grown at their optimal growth condition (Bulushi et al., 2009; Rodriguez-Jerez et al., 1994). A toxic concentration of histamine (>200 ppm) was generally observed when HPB reached the middle or end of exponential phase of their growth, although Takahashi et al. (2003) suggested that histamine accumulation occurred in the late stationary phase of *M. morganii*. This correlation, which results from the production of the *hdc* enzyme by HPB during their growth (James et al., 2013), can be further used to predict the histamine formation in specific food products at different conditions of processing or storage (Emborg & Dalgaard, 2008a).

Table 1-7. HPB counts and histamine concentration in broth and fish product

Organism	HPB counts (log CFU/ml)	Histamine (µg/ml or mg/kg)	Growth condition	Reference
M. morganii	6.41	1.97	Niven at 37°C for 24h	Rodriguez-Jerez et al. (1994)
	8.81	1,401.56		
	9.34	2,895		
	9.51	144	In histidine-TSB, incubated at 30°C for 48h	Torres et al. (2002)
R. ornitholytica strain Lc22-2	9	>850	TSBH with 0.5%NaCl, incubated at 35°C for 36h	Lin et al. (2014)
P. phosphoreum	7	100	Cold smoked salmon stored at 5°C	Jørgensen et al. (2000)
P. vulgaris	9.79	26.41	In histidine-TSB, incubated at 30°C for 48h	Torres et al. (2002)
Aeromonas spp.	8.08	5.24	In histidine-TSB, incubated at 30°C for 48h	
Pseudomonas spp.	6.23	6.44	In histidine-TSB, incubated at 30°C for 48h	
P. putrifasciens	5.1	2.45	In histidine-TSB, incubated at 30°C for 48h	
Total HPB	6.59	>40	Longtail tuna (*T. tonggol*) stored at 25°C for 18h	Mahusain et al. (2017)
	7.23	>70	Longtail tuna (*T. tonggol*) stored at 25°C for 24h	
	7.71	>100	Longtail tuna (*T. tonggol*) stored at 37°C for 18h	
	9.75	>110	Longtail tuna (*T. tonggol*) stored at 37°C for 24h	
	1	Negligible	Yellowfin tuna (*T. albacares*) fillet stored at 4, 10 and 22°C	Du et al. (2002)
	2 - 3	>50	Yellowfin tuna (*T. albacares*) fillet stored at 10 and 22°C	
	5.7	Negligible	Albacore stored at 25°C	Kim et al. (2002)
	~8	172	Albacore stored at 25°C	
	7 - 8	604	Albacore (*T. alalunga*) stored at 25°	Kim et al. (1999)

During food production, histamine accumulation mainly occurs due to time delays and temperature abuse. Although HPB grow at a wide range of temperature, the most rapid growth leading to the high Hdc activity occurs at high temperature. Freezing suppresses enzymatic activity, however, enzymatic activity still occurs slowly at refrigeration temperature (Lehane & Olley, 2000).

Furthermore, Hdc activity can also be inactivated by cooking or heating food. However, as a heat-stable amine, histamine cannot be eliminated by heat or freezing (Kim et al., 2002; US FDA, 2011). Improper evisceration and gill removal can also contribute to the transfer and spread of HPB from the gills, skin and intestinal tract of fish into the fish flesh (FAO & WHO, 2012; Visciano et al., 2012). Therefore, preventive actions such as maintaining the cold chain system, assuring hygienic practices and avoiding cross-contamination are the most effective ways to avoid histamine accumulation.

High levels of histamine are more prominent in spoiled fish. Once dead, the defence mechanism of fish no longer inhibits bacterial growth in muscle tissue, and therefore HPB can grow and produce histamine (US FDA, 2011). Spoiled fish products, however, usually have an unpleasant odour and specific appearance that make them easier to recognise as no longer suitable for human consumption. Therefore, the possibility of HFP from consumption of spoiled fish is low. On the other hand, unspoiled fish that contain high histamine levels do not have a remarkable odour and appearance, and are more likely to be consumed (Visciano et al., 2012). In addition, histamine formation occurs before post-mortem, and hence organoleptic properties of fish cannot be used to distinguish between fish with high levels of histamine versus fresh fish (Lehane & Olley, 2000). This factor leads to another difficulty to detect histamine accumulation at the earliest stage. As a mitigation approach, biogenic amine levels in seafood are used as one of the quality parameters to determine fish freshness (Veciana Nogués et al., 1997).

HFP is usually associated with fish containing more than 200 mg/kg of histamine, often above 500 – 1,000 mg/kg, thus the FDA established the safety limit of histamine at 500 mg/kg (US FDA, 2011) . Fish contains naturally high levels of histidine in their muscle tissue (including tuna, bonito, and mackerel) and foods containing these fish, are considered as spoiled and not fit for consumption if they contain more than 50 mg/kg histamine (US FDA 2005). Therefore these fish cannot be distributed if one part of the fish contains more than 50 mg/kg, as the possibility exists that other parts of fish might contain more than 50 mg/kg (Emborg & Dalgaard, 2006; Torido et al., 2014; US FDA, 2011). Species variations and the possibility of higher histamine levels in the other parts of the

fish are several aspects that have been taken into consideration. The European Commission requires a low safety level of histamine in fishery products from fish species associated with higher amounts of histidine (Reg. EC 2073/2005). Following the three-class sampling plan, amongst nine sets of samples, no more than two samples may exceed 100 mg/kg histamine (m), and no sample may exceed 200 mg/kg histamine (M) (European Commission, 2005). The National Standardization Body (NSB) of Indonesia regulates maximum histamine level of fish at 100 mg/kg (SNI 2729:2013) (Indonesian National Standardization Agency, 2013).

1.7. Histamine detection methods

1.7.1. Chromatographic-based methods

Histamine is an organic molecule with low volatility and lack of chromophores (Sagratini et al., 2012) therefore the identification of this compound is usually performed by chromatography (Onal, 2007). Thin layer chromatography (TLC) is the simplest chromatography method that does not require complex and sophisticated equipment, however, the results are inconsistent and semi-quantitative (De La Torre, 2013) with a very high histamine detection limit of 1,000 ppm (Romano et al., 2012). This method is therefore, less appropriate for histamine analysis because the US Food and Drug Administration (US FDA) has established the quality and toxicity levels of histamine for tuna, mahi-mahi and related fish at 50 and 500 mg/kg, respectively (US FDA 2005). However, TLC is still acceptable as a quick qualitative screening method for routine analysis of histamine by food authorities or industries (Onal, 2007).

Gas chromatography (GC) is less commonly used in the determination of histamine and biogenic amines due to their low volatility. However, several studies suggested that using GC can reduce analysis time (Hwang et al., 2003), and the biogenic amine levels obtained from this method are well correlated with other methods such as capillary electrophoresis and fluorometry (Du et al., 2002) which has been accepted as a reference method (AOAC 977.13).

Liquid chromatography (LC) with reverse phase (RP) (Hungerford, 2010) is currently the most preferred method to separate histamine and other biogenic amines in fish and fish products (De La Torre, 2013). Modified (non-polar) silica, with silica bound to alkyl hydrocarbon chains such as C-8 or C-18, is the stationary phase in the RP system, while the mobile phase is a mixture of polar compounds. Although polar biogenic amines can be eluted from this system, they have a weak ability to bind to the stationary column, resulting in poor retention (Ellis et al., 2009). Hydrophilic interaction chromatography (HILIC), which is based on normal-phase (NP) chromatography, was proposed to counter this problem. HILIC, previously known as aqueous normal-phase LC, uses the combination of hydrophilic stationary phase with a mixture of water-organic solvents as the mobile phase (Alpert, 1990; Jandera, 2011). These water-organic mixtures improve separation of highly polar compounds such as biogenic amines and distinguish this system from NP chromatography, where mostly only organic solvents are used (Yoshida et al., 2012).

Simultaneous detection of histamine and other biogenic amines is essential from a food safety perspective because the presence of histamine potentiators, such as agmatine, putrescine, cadaverine, anserine, spermine, and spermidine, might enhance the toxicity of histamine in fish products. However, these potentiators are usually present in very low concentrations in fish which are associated with HFP cases. Therefore the LC method is suitable to be used as this method is sensitive enough to quantify histamine and other biogenic amines that are present in low levels in food samples (Bomke et al., 2009; Sagratini et al., 2012; Veciana Nogués et al., 1997).

Another consideration for histamine analysis of food products is the effect of the food matrix during the extraction step. This matrix complexity introduces difficulties during extraction procedures where recovery rate is critical (De La Torre, 2013). Therefore, most analyses of biogenic amines include post- or pre-column derivatization to modify the polarity of molecules and increase fluorescence activity of biogenic amines, thus enhancing the sensitivity of the analysis. Post-column derivatization occurs after column separation, while in the pre-column method, biogenic amines are derivatized before being injected into the column. Reagents that are generally used to prepare the

derivatives of biogenic amines are o-phthalaldehyde (OPA), benzyl chloride, dansyl and dabsyl chloride, fluoresceine, 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC), 3-(4-chlorobenzoyl)-quinoline-2-carboxaldehyde, 9-fluorenylmethyl chloroformate, naphthalene-2,3-dicarboxaldehyde and succinimidylferrocenyl propionate (SFP) (Onal, 2007; Sagratini et al., 2012). OPA is the most popular amongst other chemicals and has been used in post-column derivatization of several biogenic amines in food (Iijima et al., 2013; Latorre-Moratalla et al., 2009) and fish products (Veciana Nogués et al., 1997), as well as in the pre-column derivatization of biogenic amines in dark and white muscle fish (Antoine et al., 2001).

Post-column derivatization, in particular, involves less sample manipulation in the preparation step (Iijima et al., 2013), and is therefore suitable for automatic sample injection and routines analysis. This method separates samples with complex matrices such as food, before the reaction, thus reducing the matrix interference during detection. Veciana Nogués et al. (1997) confirmed the lack of interference from volatile amines, amino acids and dipeptides by using post-column derivatization to detect biogenic amines in fish. However, post-column derivatization requires extensive optimisation of chromatographic conditions and the reaction system to achieve high sensitivity and reproducibility.

However, Song et al. (2004) performed pre-column derivatization of agmatine from rat tissue samples and found that the derivatives of agmatine and 7-fluoro-4-nitrobenzoxadiazole (NBD-F) were well separated from endogenous water-soluble compounds in the sample matrix, and thus improved detection sensitivity. Although it has higher sensitivity than post-column derivatization, the pre-column methods are limited to the screening of specific or purified amino acids, because the derivatization reagents might also react with the unspecified matrix of complex samples and affect separation.

For quantitative purposes in chromatography, a detector is needed to convert the properties of the targeted compounds into electrical signals that can be expressed as a compound

concentration. Different types of detectors are available for high performance liquid chromatography (HPLC) quantitation, such as ultraviolet-visible (UV-Vis), fluorescence, photo diode array (DAD), MS, infrared spectrometry and NMR.

A UV Vis detector, which measures the light absorbance of the samples, is the most popular detector. It is simple to operate and responds to different wavelengths (from 190 - 600 nm) with high sensitivity, thus is considered a universal detector (Snyder et al., 2010; Swartz, 2010). However, this detector is not suitable for compounds that do not absorb in the UV visible region. Similar to a UV-Vis detector, a fluorescence detector utilises a broad UV spectrum as the light source. However, it has a higher specificity and sensitivity than a UV detector, especially for compounds that absorb light at one wavelength and emit the light (fluorescence) at a different wavelength (Snyder et al., 2010). Photo diode array detection (DAD) is another UV-based spectrophotometer that passes reflected light from the sample into a multichannel detector to generate an analyte spectrum. With its ability to collect data from one or more wavelength across the chromatogram, DAD can be used to identify peaks based on different simultaneously read spectra (Schubring, 2010; Snyder et al., 2010). Application of these detectors to analyse histamine from different food products is presented in Table 1-8.

Table 1-8. Application of different chromatography detectors to analyse histamine from fish products

Food type	Detector	Extraction method	Sample derivatization	Wavelength	Histamine concentration	Reference
Canned tuna, bonito, mackerel and anchovy	UV Vis	6% trichloro acetic acid (TCA)	Pre-column with benzoyl chloride	254 nm	0.45 – 54 ug/g	Yen and Hsieh (1991)
Marlin fillet implicated in HFP	UV Vis	6% TCA	Pre-column with benzoyl chloride	254 nm	84.1 mg/100 g	Hwang et al. (1997)
Fresh and salted-dried mackerel, salted-dried sardine	UV Vis	5% TCA	Pre-column with dansyl chloride	254 nm	1.95 - 59.61 mg/100 g	Jeya Shakila et al. (2001)
Canned mackerel implicated in HFP and other canned mackerel of the same brand and lot	UV Vis	6% TCA	Pre-column with benzoyl chloride	254 nm	61.2 - 153.9 mg/100 g	Tsai et al. (2005b)
Sardines and anchovies stored at different temperature	UV Vis	0.4M Perchloric acid	Pre-column with dansyl chloride	254 nm	1.5 - 122.6 mg/100 g	Visciano et al. (2007)
Fish cube implicated in HFP	UV Vis	6% TCA	Pre-column with dansyl chloride	254 nm	40.02 - 52.30 mg/100 g	Chen et al. (2010a)
Skipjack tuna	Fluorescence	5% TCA	Pre-column with OPA	320 nm (ex) and 523 nm (em)	7.43 - 185.5 mg/kg	Tahmouzi et al. (2013)
Tuna (spiked)	DAD	HClO4 1M	No derivatization			Cinquina et al. (2004)

Despite the ability of pre- and post-column derivatization to increase the sensitivity of biogenic amines detection using fluorescence or UV detectors, the major drawback of these methods is peak identification, which is based on column retention time and which can cause difficulties with complex samples (Bomke et al., 2009). In addition, the level of histamine and other biogenic amines in fresh fish is usually low (0.1 – 1 mg/100 gr) (Auerswald et al., 2006; Muscarella et al., 2013) resulting in a weak fluorescence signal that can be difficult to distinguish from any signal interference (noise). This problem can be resolved by the integration of mass spectrometry (MS) as the detector, which creates ionized molecules of analytes and identifies them based on their mass to charge ratio (*m/z*).

Since the application of MS does not rely on the chromophore groups of biogenic amines, the derivatization step can be eliminated to reduce the time for analysis. The mass analyser is another important aspect of MS analysis, as this device takes the ionized molecules and separates them for later reading by the detector. The most common mass analysers are quadrupole and ion trap which are based on similar principles using an electric field to separate ions, while orbitrap and time-of–flight, separating ions by time and applying the basic principle of chromatography. For biogenic amine analysis, electrospray ionization (ESI) has been used as an ion source for several LC-MS analyses (Sagratini et al., 2012; Song et al., 2004). ESI is more suitable for molecules with moderate polarity, such as peptides, and due to low energy collision, it produces little fragmentation for faster and easier identification (Pitt, 2009). Sirocchi et al. (2014) identified ten underivatized biogenic amines in meat using LC-MS/MS and showed that the application of triple quadrupole (TQ) as mass analyser increased the sensitivity and selectivity of the analysis. However, in the MS/MS analysis, the influence of sample matrices on the ionisation process should also be evaluated by including a standard solution in the analysis (Sagratini et al., 2012).

1.7.2. Fluorometric analysis

AOAC Official Method 977.13 recommends the fluorometric method to quantify histamine in seafood. Separation using an ion exchange column followed by post-column derivatization using *o*-Phthaldialdehyde (OPA) is suggested (AOAC International, 2012). The fluorometric method is also recommended by the Indonesian NSB for histamine in fish products (SNI 2354.10:2009) . However, this method is not the best option for routine analyses, due to time and skills required.

1.7.3. Capillary electrophoresis

Capillary electrophoresis (CE) was developed to reduce the time required for biogenic amine separation in most HPLC methods, by manipulating the condition for separation to minimize the interfering peaks. Although the detection limit of CE is lower than HPLC, and not all biogenic amines can be analysed without derivatization, the time required by this method to separate biogenic amines is faster than HPLC (Gardana et al., 1999; Lange et al., 2002). Recent developments to improve the sensitivity of this method to analyse biogenic amines from different types of foods have been achieved by combining CE with different detectors, such as UV (Vitali et al., 2013), DAD (Numanoğlu et al., 2008), electrochemiluminescence (An et al., 2015) and MS (Daniel et al., 2015).

1.7.4. Colorimetric and enzymatic assay

Patange et al. (2005) developed a colorimetric assay to quantify histamine based on the reaction between the imidazole ring with *p*-phenyldiazonium sulfonate. This method produces a high correlation of the histamine concentration and the colour intensity, measured at 496 nm, and has a high recovery level (>90%) and low detection limit, at 1 mg/100 g of histamine from fish. With simple extraction steps and low operational costs, this method might be used as a rapid assay in routine examinations of seafood quality.

Recently, rapid quantification of histamine from a fish sample can also be done by using commercial kits. Ridascreen® R1605 (R-Biopharm, Germany), MaxSignal® Histamine (Bioo Scientific, USA), and Kikkoman Histamine Test (Kikkoman, Japan) measure the colour produced by the

oxidation of histamine from the addition of dehydrogenase. The amount of histamine present in the sample is correlated with the colour changes. Veratox® (Neogen, USA), Ridascreen® R1601/R1604 (R-Biopharm, Germany), HistaSure® (LDN, Germany), and EIA Histamarine (Immunotech, Prague) are based on the principle of indirect competitive ELISA between the bound (histamine) and unbound (acylated-histamine) antigens with the histamine antiserum conjugate. Some of these ELISA-based kits have been approved as AOAC performance-tested methods for fish, including Veratox® (AOAC-RI 070703) (Lupo & Mozola, 2011), HistaSure® (AOAC-RI 021402) (Manz & Booltink, 2014), and EIA Histamarine (AOAC RI 980802) (Pessatti et al., 2004). In general, the time required to perform such tests is less than other methods mentioned above with the lowest detection limit of 0.4 ppm.

1.8. Indonesian salted-boiled fish (*pindang*) industry

Indonesian *pindang* is traditionally prepared using Scombroid fish, such as tuna, mackerel and scad as raw materials, while freshwater species such as tilapia, carps and milkfish are less commonly used. The process basically combines salting and steaming or boiling which serve as preservatives. Variations on the processing of *pindang* exist, including choice of raw material, salt concentration, boiling time, and packaging materials, which all depend on the common practices and the sense of taste in societies at the different location. These variations determine the characteristics of the product, including its organoleptic appearance and shelf life (Wibowo, 1996).

According to the Indonesian NSB, there are two categories of *pindang* processing (Figure 1-1).

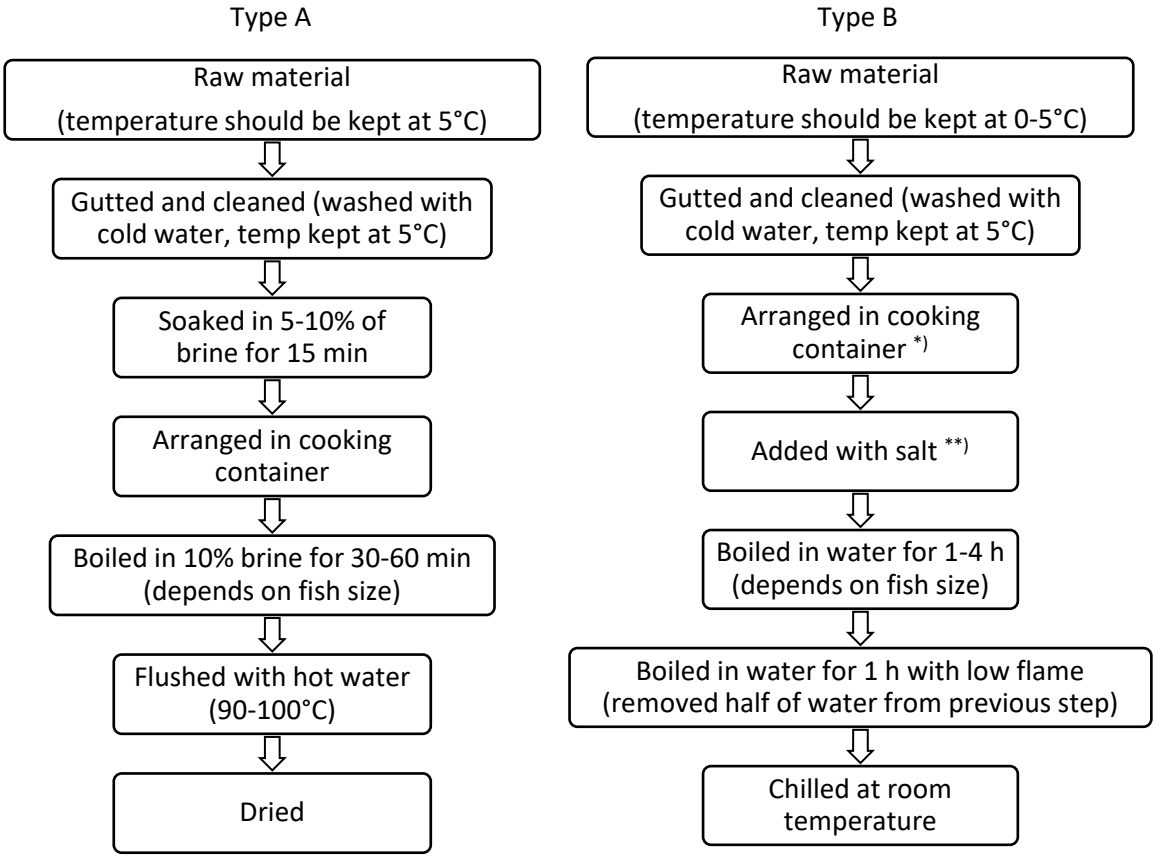

Figure 1-1. Flowchart of *pindang* processing (SNI 2717.3:2009)

*Note: *) container base is layered with salt, top layer is covered with paper, **) salt is added in between fish layers, 20-30% salt is used per kg fish*

The safety and quality requirements for *pindang* is regulated by SNI 2717.1:2009 (Table 1-9), while fresh or raw fish are regulated by SNI 2729-2013. The histamine levels of fresh or raw Scombroid fish should not exceed 100 mg/kg.

Table 1-9. The safety and quality requirement of *pindang* (SNI 2717.1:2009)

Categories	Unit	Requirements	
		***Pindang* type A**	***Pindang* type B**
A. Sensory	Scale of 1-9	Min 7	Min 7
B. Microbiological contamination			
- TVC	CFU/g	Max 5.0 x 10^5	Max 5.0 x 10^5
- *Escherichia coli*	MPN/g	Max <3	Max <3
- *Salmonella*	MPN/25 g	Negative	Negative
- *Vibrio cholerae*	MPN/25g	Negative	Negative
- *Staphylococcus aureus*	CFU/g	Max 1.0 x 10^3	Max 1.0 x 10^3
C. Chemical			
- Water content	%	Max 60	Max 60
- Salt	%	Max 10	Max 10
- Histamine	mg/kg	Max 100	Max 100

The production volume of *pindang* is the second largest amongst other traditional fish product in Indonesia, and more than 100,000 tonnes were produced in 2011 (Directorate General of Capture Fisheries, 2011). *Pindang* processors are spread over different geographical areas, including Sumatera, Java, Bali and West Nusa Tenggara. The Association of *Pindang* Processor Indonesia (APPIKANDO) estimated the number of processors was more than 65,000 in 2012 with approximately 24,000 of them located in West Java (Anonymous, 2012b). The biggest processing centres are located in Pelabuhan Ratu-Sukabumi District (West Java Province), Juwana (Central Java Province) and Klungkung (Bali Province) (Heruwati et al., 2012).

Pindang processors are family or neighbourhood-based industries equipped with basic (traditional) processing equipment, with processing techniques passed from generation to generation. The products are mostly distributed locally by food-peddlers within one to two days after processing, although some processors with greater capital distribute products to surrounding cities. The importance of this product in economic and social aspects of society, together with the increasing demands of the product from cities located further from the processing centres, indicates

the potential for *pindang* processing to be scaled to an industrial level as initiated by the Indonesian Ministry of Marine Affairs and Fisheries (Section 1.2.).

The *pindang* industry in Pelabuhan Ratu (Sukabumi District, West Java Province) was established in the 1980s. Theoretically, the processing of *pindang* in Pelabuhan Ratu can be categorised as type B processing, based on the SNI guideline 2717.3:2009. The main difference between type A and type B processing is the salting method. In type A processing, fish is soaked and boiled in 5-10% brine, then flushed with hot water after boiling. In type B processing, salt is added onto the fish surface, then fish is boiled in water and chilled after boiling.

Fish processing in this district usually depends on the local catch. However, the reduction in volumes of fresh fish being caught from the surrounding waters changes the choice of raw material used to make *pindang*, from fresh to frozen fish. In 2012, 15-20% of the total raw materials used in *pindang* processing were obtained from imported fish, most of which were frozen (Anonymous, 2012a). Since the introduction of frozen fish as raw material, there is an additional step in the processing, which is thawing. This step potentially triggers abusive temperature and time delays that promote bacterial growth and can cause elevated levels of histamine in the final product.

1.9. Strategies to control histamine formation during the processing of *pindang*

Due to the use of frozen fish as raw material, a thawing stage is introduced in the preparation phase. Jason (1974) described different methods to thaw frozen fish in fish processing facilities, *i.e.* thawing in still and moving air, as well as in water. Thawing in still air is done by laying the frozen fish block or individual fish overnight at room temperature (temperature should be kept at 18°C). This method is the most straightforward and practical for small-scale processing units, since it does not require any sophisticated equipment. However, the thawing and handling time might be longer than other methods. For example, a single 100 mm thick fish needs 8 - 10 hours to thaw. Thawing in moving air requires more complex equipment and room design, however the thawing process is faster than in still air. Fish should be arranged in such a way to obtain as large surface area as

possible. Thawing a 100 mm-thick frozen cod with 8 m/s moving air (temperature should be kept at 20°C) can be done in 4 - 4.5 hours. Thawing in water is recommended for whole frozen fish rather than filleted fish. This method can be done as long as clean water supplies, and water recirculation systems are available. The time required to perform this technique is similar to thawing in moving air. However, the *pindang* processors are mostly located in rural coastal areas around Indonesia, with limited or no access to cooling facilities, such as freezers and refrigerators. Therefore, the application of cold chain systems in the *pindang* processing, including the introduction of proper thawing methods, remains challenging.

Alternative methods to prevent histamine formation have been investigated. There have been several studies on the discovery of natural compounds that have the ability to degrade histamine or inhibit the activity of Hdc enzyme. Herbs, spices and plants have shown abilities to inhibit Hdc enzyme activity (Table 1-10). Although some of the studies were done using Hdc from mammalian cells, the property of mammalian Hdc is similar to bacterial Hdc, *i.e.* both require pyridoxal 5'-phosphate as a cofactor (Landete et al., 2007; Nitta et al., 2013). Therefore, these compounds can also be used to inhibit the Hdc activity of Gram-negative HPB.

Cloves and meadowsweet extracts applied in mackerel were able to keep the histamine levels at the concentration lower than 100 mg/kg during storage at room temperature (24 - 30°C) for 24 h (Nitta et al., 2016; Shakila et al., 1996). While green tea and black tea extract applied in fresh Little Tuna and mackerel showed a reduction of histamine levels in the treated group compared to control (Heruwati et al., 2009; Nugraha et al., 2016). Another enzyme that has been proposed to degrade histamine in fish paste (Rihaakuru) from the Maldives is DAO (Naila et al., 2014; Naila et al., 2012). In the tuna soup used to prepare Rihaakuru, DAO was able to reduce histamine levels from 500 mg/l to undetectable levels (<0.5 ppm). Other natural compounds such as chitosan, citric and lactic acids were proposed to be used as a treatment of raw fish (Ariyani & Yennie, 2008; Dwiyitno et al., 2005) since these compounds were able to inhibit the growth of HPB and reduce the levels of histamine in the final *pindang* product. Furthermore, benzoic acid (Heruwati et al., 2008) and EGCG

from green tea extract (Heruwati et al., 2009) have the ability to inhibit the activity of both the bacteria and the Hdc enzyme of Eastern Little tuna (*Euthynnus affinis*) and mackerel (*Scomber australasicus* SV).

Table 1-10. Natural compounds with the ability to inhibit Hdc enzyme activity

Compound	Source	Inhibition activity (%)	Hdc enzyme origin	Reference
Eugenol	Cloves	31 - 100	*E. aerogenes* and *M. morganii*	Ohshita et al. (2007); Shakila et al. (1996); Wendakoon and Sakaguchi (1995)
Cinnamic aldehyde	Cinnamon	90 - 100	*E. aerogenes* and *M. morganii*	Ohshita et al. (2007); Wendakoon and Sakaguchi (1995),
Ellagitannins	Meadowsweet	50 - >90	Recombinant human *hdc* and *M. morganii*	Nitta et al. (2013); Nitta et al. (2016)
Quercetin glycoside	Allspice	55 - 64	Recombinant human *hdc*	Nitta et al. (2009)
Epigallocatechin gallate (EGCG)	Green tea	57 - 67	Recombinant and natural rat *hdc*	Rodriguez-Caso et al. (2003)

The use of herbs, spices and other natural compounds as pre-treatment during the processing of *pindang* becomes another potential strategy to reduce histamine levels in the products, as they are relatively cheap and easy to get. Herbs and spice could also enhance the product flavour.

Apart from maintaining cool temperature during *pindang* processing, unhygienic practices were also identified as a critical control point. Although salt addition and cooking should be adequate to eliminate the bacteria from raw fish, however unhygienic practices during handling, storage and distribution of cooked fish may cause post-processing contamination. Different HPB have been isolated from pindang, including *P. vulgaris, H. alvei, M. morganii, E. aerogenes, K.*

oxytoca, K. pneumonieae (Fatuni et al., 2014), showing the presence of post-processing contamination because these bacteria should not survive the boiling process.

Improving the *pindang* packaging could be another intervention strategy to prevent *post*-processing bacterial contamination. Vacuum packaging (VP) has been introduced as an alternative to replace paper that is currently used to pack *pindang* (Kristianto et al., 2017; Purwaningrum, 2014). The shelf-life of vacuum-packed *pindang* stored at room temperature was nine days longer than the paper-wrapped fish (Kristianto et al., 2017). VP was also used in soft-bone milkfish, another traditional fish product from Indonesia. The vacuum-packed milkfish has a shelf-life of 1 month at room temperature (Susanto, 2010). However, several HPB are facultative anaerobes and able to grow in vacuum conditions. Therefore VP cannot be used as the sole approach to inhibit histamine formation (US FDA, 2011). A combination of vacuum-packaging with low storage temperature is proposed as a better approach to prevent histamine formation and deterioration of fish (Kung et al., 2017).

1.10. Microbiological risk assessment (MRA)

Within the risk analysis framework, a risk profile was performed as part of the risk analysis preliminary activities, where information on the relevant data including hazard-food commodity combination of concern, description of public health problem, as well as information on food production, processing, distribution and consumption were collected and used as guidance to perform a risk assessment (FAO & WHO, 2006). A risk profile should also aim to identify the major knowledge gaps of the problem which will then be addressed in the risk assessment.

Risk assessment is the scientific element of food safety risk analysis which aims to identify ways to protect consumers from eating contaminated foods. It should be performed in a structured approach, *i.e.* including the steps of 'hazard identification', 'hazard characterisation', 'exposure assessment' and 'risk characterisation', as defined by the Codex Alimentarius Commission (Joint FAO/WHO, 1999).

Risk assessment (RA) is categorized into qualitative, semi-quantitative and quantitative risk assessment (Sumner et al., 2004). Qualitative RA is the most straightforward but more subjective approach which is usually performed as an initial attempt to evaluate risk and provide risk managers with evidence of the importance of further quantitative assessment for a particular risk. The output of qualitative RA can also be used as a recommendation whether necessary measurements have to be taken during the food processing to minimise the risk or reduce the hazard (FAO & WHO, 2009b). Qualitative RA uses descriptive categories obtained from literature review and expert consultation to describe risk and does not require sophisticated mathematical equations. Table 1-11 provides an example of how risks can be described qualitatively.

Table 1-11. Examples of qualitative descriptions of risk

Risk term	Definition			
	Clough et al. (2006)	**Horigan et al. (2014)**	**Based on the probability of event per year (FAO, 2009b)**	**Based on the exposure per year (FAO, 2009b)**
Negligible	The event is extremely unlikely to occur	So rare that it does not merit to be considered	Indistinguishable from 0	Indistinguishable from 0
Very low	-	Unlikely to occur	$< 10^{-4}$, except 0	1-2
Low	The event may occur occasionally	Rare, but may occur occasionally	10^{-3} to 10^{-4}	3-10
Medium/ Moderate	The event will occur reasonably often	Occurs regularly	10^{-2} to 10^{-3}	10-20
High	The event is very likely to occur	Occurs very regularly	10^{-1} to 10^{-2}	20-50
Very high	-	Is almost certain to occur	$> 10^{-1}$, not 1	> 50
Certain	-	-	1	-

Qualitative RA has been used to evaluate microbiological risk from a variety of foods. Gonzalez et al. (2011) performed a qualitative RA on the risk of foodborne diseases from consumption of Swiss dairy products. The products and the associated microbial hazards, including *Campylobacter* spp., *Listeria monocytogenes*, *Salmonella* spp., Shiga toxin-producing *Escherichia coli*, coagulase-positive staphylococci and *Staphylococcus aureus* enterotoxin, were determined based on a risk profile. The overall probability, obtained as a combination of release and exposure assessment, was used to estimate the likelihood of products exceeding the maximum regulatory limit and being consumed. Although the risk could not be quantified due to the lack of data, the output of this study could be used as a recommendation to design a risk-based monitoring program of the dairy product in Switzerland. Clough et al. (2006) and Horigan et al. (2014) also applied qualitative RA to define the risk of microbial contamination from consuming small game birds in the UK and verocytotoxigenic *E. coli* (VTEC) from on- and off-farm dairy products. Mihajlovic et al. (2013) used a similar approach to assess the risk of foodborne outbreaks that were linked to unpasteurized fruit juice and cider. That study used literature and database research to determine the current knowledge gaps on the probability of pathogenic contamination during the juice and cider processing. Further evidence on the effectiveness of different measures to control contamination during processing was also evaluated and used to support the risk management decision.

To perform a microbiological risk assessment, available data on microbial contamination and the risk probability are not always sufficient, because the human responses are also dependent on the number of microorganisms present in foods, especially pathogenic microorganisms (FAO & WHO, 2009b). Furthermore, without clear and quantifiable explanation of each category, risk categories might lead to misperception of the risk and cause inconsistency in the decision making.

A semi-quantitative approach combines descriptive analysis with specific categories and numerical values and offers more precise risk description of a particular microbiological hazard. Sumner and Ross (2002) applied a semi-quantitative risk assessment to rank the risk of ten Australian seafood hazard/product combinations and used the data to assist risk evaluation by the

managers. A semi-quantitative risk assessment spreadsheet was used to pair hazard and product; then the risks were categorised into different scales based on the available literature and expert opinion. The combinations (pairing) between probabilities of illness per day and predicted cases per year in a specific community were used to generate three different risk categories of Australian seafood. Ranking < 32 was correlated with hazard/products pairings that have not been related to any foodborne illness, such as mercury poisoning, *Clostridium botulinum* in canned fish and vacuum packed cold-smoked fish, viruses in shellfish from uncontaminated water, enteric bacteria in imported cooked shrimp and algal biotoxin from controlled waters. Rankings 32-48 were correlated with hazard/products pairings that have caused several outbreaks of food poisoning in Australasia, such as listeriosis outbreak from smoked mussels, *Vibrio* spp contamination in oyster and shrimp, as well as ciguatera and scombroid poisoning. The highest category in the rankings were those >48. All products/hazards pairs that belong to this category have caused food poisoning incidences, including ciguatera from recreational fishing in susceptible areas, viruses in shellfish from contaminated water and algal biotoxins (Sirocchi et al., 2014).

A quantitative approach of microbiological RA constructs mathematical models to help understand the complex nature and responses of microorganisms in the food chain system (Lammerding & Paoli, 1997). To perform a quantitative RA, a baseline risk of illness in a given population should first be determined in the process. Once the baseline risk has been determined, factors that influence the presence of specific microorganisms in food can be evaluated, and the best intervention strategies can be decided and applied into the food production system (Forsythe, 2002; Song et al., 2004). Any predicted improvement from applying the interventions can be used to help food managers or decision makers in deciding the most suitable and beneficial strategies to minimise foodborne illness (FAO & WHO, 2009b).

Quantitative RA can be categorised into deterministic and probabilistic (stochastic) categories (FAO & WHO, 2009b; Lammerding & Fazil, 2000). The deterministic approach uses a single value, such as an average value or the worst case scenario, as an input in the assessment. While the

probabilistic approach uses any available data to develop the distribution of probability and to describe the parameters that contribute to the risk (Lammerding & Fazil, 2000). The probabilistic approach is the most common one to be used in the quantitative microbiological RA. As an example, this approach was used to estimate the risk from listeriosis from some ready to eat foods, including fluid milk, ice cream, semi-dry fermented meats and cold-smoked fish (Food And Agriculture Organization & World Health Organization, 2005). A mathematical model was used to determine the risk per serving as well as the risk in a population per year. The study found data that showed the probability of listeriosis depends on the food matrix, the virulence of the *Listeria* strain and the susceptibility of the consumers. The risk modelling suggested that nearly all of the listeriosis cases are due to the consumption of high numbers of *L. monocytogenes* and that control measures to prevent any *Listeria* contamination in foods, or further growth in foods, are expected to have the highest impact on reducing the cases of listeriosis. Another example is the risk assessment of *Vibrio parahaemolyticus* in bloody clams in southern Thailand (Yamamoto et al., 2008). A model showing the production-to-consumption framework of bloody clams, which consists of four major steps, *i.e.* harvesting, retail, cooking and consumption, was built in this study. The estimated risk of illness due to the consumption of bloody clam contaminated with pathogenic *V. parahaemolyticus* was estimated as 5.6×10^{-4} times per person per year. Furthermore, a sensitivity analysis of consumer behaviour showed that improper cooking greatly affects the probability of illness. Although there were limitations on the quantitative data and several assumptions were made, this study was able to demonstrate a complete set of data collection and stochastic analysis process, and then produced risk-related results that could be used by the decision makers.

Quantitative risk assessment can be incorporated to the Hazard Analysis Critical Control Points (HACCP), a risk management tool in a food production system which is aimed to ensure food product safety from farm to fork. The first step of HACCP implementation is hazard analysis to gather and evaluate available information about any potential contaminants and to identify the most critical hazards which may cause food safety issues (USDA-NACMCF, Hoornstra et al., 2001; 1997). Based on

this information, control measures, which then applied as the critical control points (CCPs), are identified to prevent contamination, unacceptable increase of contaminants as well as to reduce the contamination to acceptable levels (Hoornstra et al., 2001). However, this management system has several limitations, such as the subjectivity during CCPs determination and the assessment of CCPs which often restricted to a qualitative analysis, the inability to quantify potential combined effects of multiple CCP variations and the variations of food products and food processing conditions (Buchanan & Whiting, 1998; Hoornstra et al., 2001; Notermans & Mead, 1996). Therefore, to provide more accurate information for the risk management, a quantitative determination of hazards associated with food consumption is needed. For example, the acceptable level of microbiological contaminant after processing can be determined quantitatively based on predictive models, microbial challenges or storage tests which are designed at specific processing condition (Buchanan & Whiting, 1998; Notermans & Mead, 1996).

1.11. Predictive modelling

Predictive microbiology is a systematic approach that can assist in the exposure assessment phase of risk assessment, to evaluate the microbiological safety and quality of food by using mathematical models to simplify the effects of complex food supply chains on the potential for growth or inactivation of microorganisms of interest (Devlieghere et al., 2006; van Boekel & Zwietering, 2007). This approach integrates science-based knowledge of microbial behaviour with practical applications to food processing. It is assumed that the responses of the microbial populations to environmental conditions are reproducible so that similar microorganisms behave similarly under the same conditions (Tamplin, 2005). Therefore, the responses of the microbial population towards different factors that support or limit their growth can be estimated or predicted over time or during food processing, distribution, storage and before consumption (De La Torre, 2013; McMeekin et al., 2007; Ross et al., 2000). Lag time, generation time, maximum growth rate

and maximum cell concentration are kinetic parameters estimated by predictive models (Skinner et al., 1994).

Buchanan (1993) divided models in predictive modelling into three different categories, which are primary, secondary and tertiary models. Primary models describe microbial responses, such as growth, death or survival, as a function of time for a specific environmental condition, while secondary models combine data from primary models to define the effect of environment on microbial growth or inactivation and produce mathematical equations. A primary and secondary model that are integrated and implemented into user-friendly software is known as a tertiary model.

As classified by Monod (1949), the bacterial growth phases consist of lag, acceleration, exponential, retardation, stationary and decline phase. However, the acceleration and retardation phases are often suppressed or very short and unobservable. Therefore, a typical bacterial growth curve is shown in Figure 1-2. Sigmoidal functions, such as Gompertz, logistic and Baranyi equations, are the most widely used primary model to determine the microbial growth because these models consist of four phases that are similar to the microbial growth phases (Skinner et al., 1994).

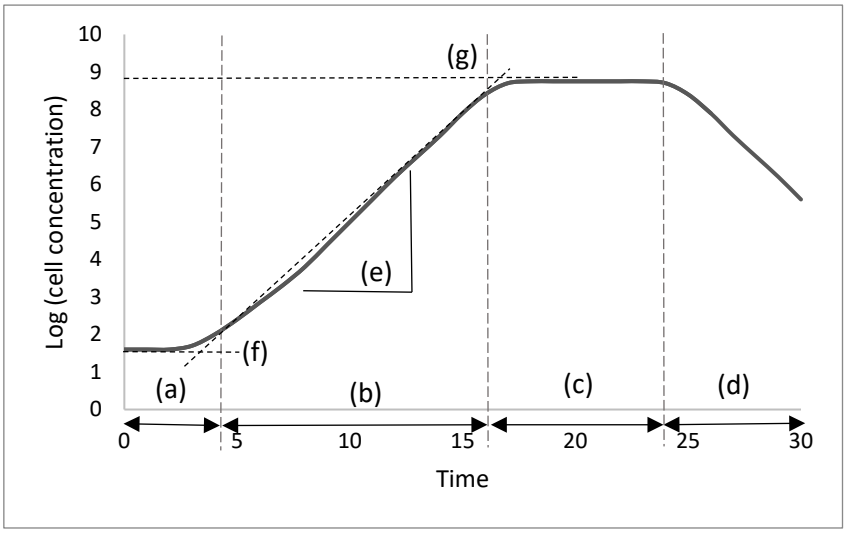

Figure 1-2. A typical bacterial growth that consists of (a) lag; (b) exponential; (c) stationary, and (d) death phase. The bacterial growth kinetic parameters are (e) maximum growth rate (μ_{max}) (f) initial cell concentration (N_0) and (g) maximum cell concentration (N_{max}).

During the lag phase (λ), bacterial cells make some necessary adjustment of their metabolic activities to adapt to a new environment, thus no growth occurs during this phase. While in the exponential phase, bacteria start to grow and to divide exponentially at a constant rate. At the end of the exponential phase, nutrient availability is depleted as it has been consumed by the bacteria. The cells then enter a stationary phase where no more growth is observed. Finally, the death phase occurs, in which the metabolic waste is accumulated, and the number of viable cells decreases exponentially (Baranyi & Roberts, 2000; Navarro Llorens et al., 2010).

In the bacterial growth model, μ_{max} is defined as the maximal value of the specific growth rate, observed as the straight line of an exponential phase (Baranyi & Roberts, 2000; Devlieghere et al., 2006; Zwietering et al., 1990). The mathematical models below are generally used to describe bacterial growth and to evaluate the environmental effect on the growth.

1. Gompertz model (Gibson et al., 1987)

   $$log\, N(t) = A + Cexp\{-exp[-B(t-M)]\}$$

2. Logistic model (Gibson et al., 1987)

   $$log\, N(t) = \frac{A + C}{1 + exp[-B(t-M)]}$$

3. Baranyi model (Baranyi et al., 1993)

   $$\frac{dN(t)}{dt} = \frac{q(t)}{1+q(t)} N(t) \left\{ 1 - \left[\frac{N(t)}{N_{max}} \right]^m \right\}$$

4. Three-phase linear (Buchanan et al., 1997)

   Lag phase ($t \leq t_{lag}$), $N_t = N_0$

   Exponential growth phase ($t_{lag} < t < t_{max}$), $N_t = N_0 + \mu(t - t_{lag})$

   Stationary phase ($t \geq t_{max}$), $N_t = N_{max}$

5. Arrhenius (Ratkowsky et al., 1982)

$$k = Ae^{\frac{-E}{RT}}$$

6. Square-root (Ratkowsky et al., 1982)

$$\sqrt{\mu} = b\ (T - T_0)$$

7. Extended Ratkowsky (McMeekin et al., 1987)

$$\sqrt{\mu} = b\ (T - T_{min})\sqrt{(a_w - a_{wmin})(pH - pH_{min)}}$$

Where $N(t)$ is the cell density (log) at time *t*, N_{max} is the final population density (log). For Gompertz and Logistic models, *A* is the value of lower asymptote (N_0or the initial population density in log), *C* is the asymptotic amount of growth that occurs as *t* increase indefinitely, *M* is the time when the maximal growth rate achieved, and *B* is the relative growth rate. For Baranyi model, $dq(t)/d(t)$ equals to $\mu_{max}q(t)$, $q(t)$ is the concentration of limiting substrate $\mu_{max}$is the maximum specific growth rate (h^{-1}), and *m* is a constant which frequently assumed as 1. For three-phase linear model, t_{lag} is the time when the lag phase ends and t_{max} is the time when the exponential phase ends. For the Arrhenius equation, k is the constant, A is the collision or frequency factor, E is the activation energy (kJ/mol), R is the universal gas constant, and T is the absolute temperature. For square root models, *b* is the slope of the regression line, T is the temperature, T_0 orT_{min},a_{wmin} and $pH_{\min}$ is a conceptual temperature, water activity and pH, respectively, where the growth rate is zero.

The Gompertz model is an empirical model that describes the observed data into the best-fitted mathematical equation, while the Baranyi model is a mechanistic model that includes the biochemical process of the lag phase into the model and the concept of strict exponential growth of bacterial populations. The bacterial cells perform biochemical reactions as a process of adjustment to a new environment and to support their growth. Once the cells have adapted, the growth will continue exponentially until no more substrate is available for growth (McKellar & Lu, 2004). As a

simpler model, the three-phase linear model describes no bacterial replication or growth during the lag and stationary phase, therefore the specific growth rate value is zero, while during the exponential phase, the cell population increase log-linearly with time. The model separates between the lag phase and adjustment periods, and it considers the behaviour of individual cells during the adaptation stage and the bacterial population as factors that affect the bacterial growth kinetics (Buchanan et al., 1997).

Secondary models describe the effect of intrinsic and extrinsic factors of food on bacterial growth responses (Devlieghere et al., 2006). The Arrhenius, Belehradek (square root), cardinal value (Rosso et al., 1995) and polynomial models are the most popular models to be used as secondary models. Arrhenius model defines a concept that the constant rate of specific chemical reaction depends on temperature. However, this model is inadequate to describe the effect of full biokinetic temperature range on the bacterial growth (Ratkowsky et al., 1982; Ross & Dalgaard, 2004). A square root (Belehradek) model that described a linear relationship between temperatures and bacterial growth, under conditions where the temperature was the only limiting factor was introduced by Ratkowsky et al. (1982). Further development of this model was proposed by (McMeekin et al., 1987; McMeekin et al., 1992) that included several other parameters, including salt concentration/water activity and pH, into the model. These square root models are probably the most important secondary models within predictive microbiology (Ross & Dalgaard, 2004).

Biogenic amines are formed as a result of bacterial metabolic activity in amino acid-rich media or matrices. The formation of biogenic amines depends on the number of bacterial cells (Rodriguez-Jerez et al., 1994), therefore, a yield factor (Equation 1-1) can be used to correlate the absolute rate of biogenic formation such as histamine (Emborg & Dalgaard, 2008a, 2008b; James et al., 2013; Jorgensen et al., 2000), tyramine (Bunkova et al., 2011), putrescine and cadaverine (Bubelová et al., 2015), to the HPB absolute growth rate (Equation 1-2). Furthermore, this yield

factor was combined with the expanded logistic growth model as a primary model for histamine formation by *M. psychrotolerans* during storage (Emborg & Dalgaard, 2008a, 2008b).

$$pY_{His/CFU} = -log\left(\frac{His(final)-His(t_0)}{CFU(final)-CFU(t_0)}\right) \quad \text{(Equation 1-1)}$$

$$His(t) = His(t_0) + Y_{His/CFU}\,x\big(CFU(t) - CFU(t_0)\big)x1000 \quad \text{(Equation 1-2)}$$

where:

$Y_{His/CFU}$ is the constant yield factor. *CFU* (t_0), $CFU(final)$ and $CFU(t)$, are colony forming units per ml at initial, final and time (t), respectively. $His(t_0)$ and $His(final)$ and $His\ (t)$ are histamine concentration in µg/ml at initial time, final time, and time (t), respectively.

Other studies described the effect of environmental factors (temperature, pH, NaCl, lactose, aerobic and anaerobic condition) on the formation of biogenic amines by using the Gompertz modified model (Equation 1-3) to predict the formation of tyramine (Bunkova et al., 2011), putrescine and cadaverine (Bubelová et al., 2015). These studies also used the yield factor equation (Equation 1-1) to combine the bacterial absolute growth at the defined condition with the histamine concentration.

$$y(t) = A_{BA}exp\{-exp\left[\frac{\mu_{BA}.e}{A_{BA}}(\lambda_{BA} - t\right) + 1]\} \quad \text{(Equation 1-3)}$$

where:

$y(t)$ is the production of biogenic amines at time$t$, μ is the maximum production rate (mg/l/h), λ is the lag time (h), and A is asymptote which is defined as maximum concentration of histamine (mg/l).

The bacterial death phase is not included in the predictive modelling of HPB and histamine formation of the fish product because this phase is usually observed when seafood products are spoiled and unfit for consumption, thus is not crucial for HFP (Emborg, 2007) and also because, once formed, histamine is not readily degraded.

Predictive modelling has been applied in other studies of the growth and histamine formation by HPB in different fish products. Bermejo et al. (2004) used a kinetic model for predicting the bacterial growth in jack mackerel (*Trachurus symmetricus*) used in a fishmeal industry, while histamine accumulation was estimated using a yield concept. The model was able to predict the behaviour of histamine during different storage conditions of the mackerel, without affecting the fishmeal quality. Using the similar concept of yield approach, Emborg and Dalgaard (2008a) predicted the growth and histamine formation of *M. psychrotolerans* in seafood at various combinations of storage (temperature and CO_2) and product characteristics (NaCl/a_w and pH). Although not highly accurate, the model provided rapid and reliable information on the likelihood of histamine formation by *M. psychrotolerans* based on its initial concentration and different product characteristics. Another application of a mathematical inactivation model was to predict the effectiveness of DAO in a tuna soup production process (Naila et al., 2012). This study observed the effect of DAO on the rates of histamine degradation and proposed the most suitable amount of DAO to be applied in the tuna soup processing to reduce the risk of histamine accumulation.

In general, a better understanding of microbial changes under dynamic environmental conditions enhances our ability to evaluate the most critical points in food processing, control or improve the established system, and support decision making in food safety management (Buchanan, 1993; De La Torre, 2013; McMeekin et al., 2007; Tamplin, 2005).

1.12. Thesis objectives

As the second biggest traditional fish product in Indonesia, *pindang* has important economic and social impacts for the Indonesian societies, especially those near the coastal areas. In different provinces in Indonesia, it is also popular as a tasty and cheap fish product which can be served in various dishes. However, *pindang* has caused many foodborne intoxications due to high levels of histamine in the final product. Unhygienic processing conditions, limited access to refrigeration

facilities and low quality of raw fish used to make *pindang*, are suggested as factors causing safety issues and low-quality levels of *pindang*.

This study aims to provide a better understanding of the behaviour of HPB and how histamine is produced during the processing of *pindang*, so that intervention strategies can be identified and applied to improve the safety and quality of the product.

The specific objectives of this study are:

1. To isolate and identify HPB from *pindang* and to assess their relative ability to produce histamine.
2. To investigate and be able to predict the bacterial growth and histamine formation under conditions that mimic the processing of *pindang*.
3. To study the changes of microbial communities of fish at different *pindang* processing stages.
4. To identify intervention strategies to improve the processing of HPB that are applicable in real processing conditions.

Chapter 2. Identification of Histamine-Producing Bacteria during the Processing of Indonesian Salted-Boiled Fish

2.1. Introduction

Histamine fish poisoning (HFP) is a mild and self-limiting disease caused by the ingestion of fish or other foods containing high levels of histamine, which is formed due to bacterial growth resulting from the combined influences of temperature abuse and mishandling of fish during processing, storage and distribution (Baylis, 2006; FAO & WHO, 2012; Hungerford, 2010; Taylor, 1986; Taylor et al., 1989). Fish containing naturally high levels of histidine in muscle tissue (including tuna, bonito, and mackerel) and foods containing these fish, are considered as spoiled and not fit for consumption if they contain more than 50 mg/kg histamine (US FDA, 2005), while those with histamine levels ≥ 500 mg/kg are considered as unsafe for consumption and might cause adverse health effects to the consumer (US FDA, 2011) . Most cases of HFP are associated with fish containing more than 500 mg/kg histamine (Feldman et al., 2005; Knope et al., 2014; McCormick et al., 2013; Scoging, 1998; Tsai et al., 2005b), although some reports suggest that a lower concentration can sometimes cause illness (Knope et al., 2014; Scoging, 1998).

Scombroid fish, such as tuna and mackerel, are the most popular fish processed as *pindang* in Indonesia, a traditionally processed fish which requires salting and steaming during processing. The *pindang* industry is one of the largest traditional fish processing industries in Indonesia and involved more than 65,000 small and medium scale processing units in 2012 (Anonymous, 2012b). The number of processing and marketing labourers in the Indonesian fish processing industry exceeded six million people in 2012 (Working Group of Marine and Fisheries Data Arrangement, 2013).

In tropical regions, salt addition to foods aims to preserve the food by reducing the water activity of the food, causing osmotic stress in microbial cells that leads to cell death or slow growth,

as well as flavouring the product (Henney et al., 2010). In the processing of *pindang*, heating and salting reduce the presence of HPB, but does not reduce the level of histamine that has been formed prior to processing. Since histamine is a heat-stable amine, it cannot be eliminated by subsequent heating or freezing (Kim et al., 2002; US FDA, 2011).

Since 2012, 15-20% of the total raw materials used in this process in Indonesia were obtained from imported fish, most of which is frozen (Anonymous, 2012a). In Sukabumi, the availability of fresh pelagic fish, such as tuna, caught from local waters and sold in local fishing ports is limited due to the seasonal variability (Anonymous, 2012a; Deswati & Muhadjir, 2015). Prior to cooking, the frozen fish needs to be thawed properly. When the thawing step is uncontrolled, or frankly abusive, histidine decarboxylase produced by bacterial growth may form histamine (Kim et al., 2002; US FDA, 2011). Furthermore, HPB that are typically only 1% of the total surface microbiota of live fish can multiply as a result of delays during fish handling, processing, distribution and lead to higher loads of histidine decarboxylase (Lehane & Olley, 2000).

Most reported HFP cases involve temperature abuse or poor hygienic conditions during the processing, storage and distribution of fish (Davis et al., 2007; Health Protection Scotland, 2010; McCormick et al., 2013). In Indonesia, the consumption of *pindang* is one of the major causes of HFP. Cases that affect a large number of people in a community or population are usually recorded by the health care facilities and are exposed by local media, while cases which involve individuals remain mostly unreported. HFP outbreaks have occurred in different provinces in Indonesia, including Yogyakarta (Anonymous, 2004), Bali (Anonymous, 2008), West Java (Anonymous, 2014, 2016) and East Java (Ainun, 2015).

Because HFP is a common food safety issue, many studies have investigated strategies to eliminate, prevent or reduce histamine accumulation in Indonesian *pindang*. In terms of inhibiting the growth of HPB and reducing the level of histamine in the final products, addition of chitosan (Ariyani & Yennie, 2008), citric and lactic acids (Dwiyitno et al., 2005) and activated charcoal

(Subaryono et al., 2004) showed beneficial effects. Other studies, which focused on *hdc*-producing bacteria and Hdc enzyme activities, showed that benzoic acid (Heruwati et al., 2008) and epigallocathecin gallat (EGCG) from green tea extract (Heruwati et al., 2009) were able to inhibit the activity of both the bacteria and the enzyme. In those studies, processing of *pindang* as the test material was done in a controlled environment, using temperatures rarely achieved in commercial processing plants. Thus the results might not represent the conditions of histamine contamination in the actual product.

This study aimed to isolate and identify high histamine-producing bacteria from Indonesian *pindang* collected from local processors in Sukabumi, Indonesia using their routine processing techniques. The histamine-producing ability of the isolates was assessed to contribute to the assessment of the risk associated with histamine accumulation in the final product, as consumed.

2.2. Material and methods

2.2.1. Fish sample collection

Pindang was prepared using Longtail tuna (*Thunnus* sp.) and Eastern Little tuna (*Euthynnus* sp.) as raw materials. Fish (raw, washed, thawed and cooked) (Table 2-1) were collected from traditional fish processors in Pelabuhan Ratu, Sukabumi District, West Java Province, Indonesia and kept cold or frozen during transportation. Longtail tuna were caught on a single day of fishing in local waters and were processed while fresh, while the Eastern Little tuna were obtained from fish suppliers in frozen form.

Table 2-1. Sampling point and sample description

Fish source	Sampling points	Sample terms
Fresh Longtail tuna	Raw fish, fresh upon arrival	Raw-local
	Fish after washing	Washed-local
	Fish after salting and boiling	Cooked-local
Frozen Eastern little tuna	Raw fish, frozen upon arrival	Raw-imported
	Fish after thawing and washing	Thawed-imported
	Fish after salting and boiling	Cooked-imported

2.2.2. Screening of histamine-producing bacteria

Bacterial isolation followed the procedures described by Torido et al. (2014) with modification of psychrotrophic incubation temperature, *i.e*., due to the instrument unavailability, the incubation of psychrotrophic isolates was done at 17°C. Prior to isolation, 5 g fish tissue was incubated in 45 ml of histidine broth medium containing 1% bacteriological peptone (Oxoid, UK), 0.3% yeast extract (Oxoid, UK), 1.5% sodium chloride (Merck, USA) and enriched with 0.5% of L-histidine (Sigma, USA). Bacteria were incubated at 30°C for 24 h for mesophilic and at 17°C for 72 h for psychrotrophic HPB species. One ml of enriched culture was then transferred into 9 ml of fresh histidine broth and incubated under the same conditions.

After incubation, 100 µl of the broth culture was streaked onto Niven agar (Niven et al., 1981) containing 0.5% tryptone (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 0.5% sodium chloride (Merck, USA), 2.7% L-histidine (Sigma, USA), 2% agar, 0.006% bromcresol purple with pH adjusted to 5.5 – 5.7. Plates were incubated at 30°C for 24 h for mesophilic and at 17°C for 72 h for psychrotrophic HPB. Typical HPB colonies that were a purple-greyish colour with or without media colour change were streaked onto tryptone soy agar (TSA) (Oxoid, UK) containing 2% (w/v) of sodium chloride (Merck, USA), and incubated at the same time and temperature conditions as above to isolate single colonies of HPB for DNA extraction.

Qualitative assays to identify HPB from isolates positive in Niven agar were done by growing the isolates in 96-well plates of histidine decarboxylase broth (HD broth) incubated at 30°C for 24 h and at 17°C for 72 h. HD broth contained (per litre): 2 g peptone (Oxoid, UK), 1 g Lab-Lemco powder (Oxoid, UK), 5 g NaCl (Merck, Germany), 10 g L-histidine (Sigma, USA), 10 ml of 0.1% bromcresol green solution, 10 ml of 0.2% of chlorophenol red solution and had pH adjusted to 5.3 (Yamani & Untermann, 1985). Presumptive histidine decarboxylase - positive bacteria were identified by colour change from light green to violet.

Isolates that returned positive results in both the Niven agar and HD broth tests were further examined by screening for the *hdc* gene. Bacterial DNA was extracted using the Isolate II Genomic DNA Kit (Bioline, AU) according to the manufacture's protocol. *hdc*-F (5'-TCH ATY ARY AAC TGY GGT GAC TGG RG-3') and *hdc*-R (5'-CCC ACA KCA TBA RWG GDG TRT GRC C-3') primer pairs which target a 709-bp fragment were used in the PCR reaction (Takahashi et al., 2003). The reaction was performed in 50 µl reaction mixtures containing 25 µl of MyTaq™ HS Mix (Bioline, AU), 20 pmoles of each primer (Geneworks, AU), 10 ng of template DNA and nuclease-free water using the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 1 min) and final extension at 72°C for 4 min. PCR products were separated in 1.5% agarose gel (Promega, AU) in 1x Tris-borate-EDTA (TBE) buffer at 90V for 50 min and visualized with a Gel Doc™ XR Imaging System (BIO-RAD, AU).

2.2.3. Histamine measurement

Approximately half of the isolates positive in *hdc* gene screening were chosen at random and tested for their ability to produce histamine using the quantitative HistaSure™ ELISA Fast Track kit (LDN Labor Diagnostika Nord GmbH & Co. KG, Germany), which applies indirect competitive ELISA allowing binding competition between the bound (histamine) and unbound (acylated-histamine) antigen with the histamine antiserum conjugate. Mesophilic and psychrotrophc isolates were grown in histidine broth at 30°C for 24 h and 15°C for 72 h, respectively. *Morganella morganii* (32) and

Morganella psychrotolerans (FE 3_12) from the Tasmanian Institute of Agriculture (TIA) culture collection were used as reference isolates for mesophilic and psychrotrophic incubation conditions, respectively. Prior to histamine analysis, a 1.5 ml aliquot of culture was transferred into micro centrifuge tubes and centrifuged at 10,000 *g* for 2 min at room temperature. Up to 1 ml of the supernatant was then filtered using 0.2 µm sterile filters (Corning, Germany) and placed into new and clean microtubes for histamine analysis. Samples were acylated prior to the quantification. The ELISA test was performed according to the manufacture's protocol. Six histamine standard solutions (0, 0.12, 0.4, 1.2, 4 and 12 ppm) with the absorbance value of each measured at 450 nm, were used to derive a calibration curve. The 4-parameter logistic (4PL) non-linear regression model (Cox et al., 2004) of the calibration curve was used to calculate the measured histamine concentration.

Histamine was also tested from representative samples of raw and cooked fish using HPLC (SNI 2354.10:2009) . Fish flesh (50 g) was ground with a blender and treated with 100 ml of 10% trichloroacetid acid ($C_2HCl_3O_2$) and centrifuged at 3,000 rpm for 10 min to remove any suspended particulates. Pre-column derivatization was done using o-phtalaldehyde (OPA). Separation was performed using a C18 column in gradient elution with acetonitrile:sodium dihydrogen sulphate (30:70) as the mobile phase. The amount of histamine was quantified by fluorescense at 350 nm (excitation) and 450 nm (emission). Significant differences ($P<0.01$) between the histamine concentration were analysed using analysis of variance (ANOVA) in Real Statistic Excel add-ins (http://www.real-statistics.com/).

2.2.4. Identification of histamine-producing bacteria

Histamine-producing isolates were randomly selected for identification using the API® 20E biochemical detection system and sequencing of the 16S rRNA gene. Identification with the API® 20E detection system (Biomereux, France) was performed according to the manufacture's protocol to confirm the identification of *Enterobacteriaceae* with single colonies of HPB grown overnight in TSA. Catalase and oxidase tests were performed to complete organism identification. Identification was

based on the numerical profile of each test determined using the Analytical Profile Index databases (V4.1).

Isolates able to produce histamine were further identified by sequencing of the 16S rRNA gene using the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). Forty microliters of each reaction mixture contained 20 µL of MyTaq™ HS Mix (Bioline, AU), 8 pmoles of each primer (Genework, AU) and 2.4 µL of template DNA. The PCR amplification was started with initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min and final extension at 72°C for 1.5 min (Bjornsdottir-Butler et al., 2011b). PCR products were cleaned using the Ultra Clean PCR clean up kit (MOBIO™, AU) and sent to the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, AU) for sequencing.

Sequences were corrected using the BioEdit Sequence Alignment Editor (Hall, 1999) in any noisy areas or areas of poor resolution. Appropriate IUB codes were inserted for double traces. The SEQMATCH tool of the Ribosomal Database Project (http://rdp.cme.msu.edu/) combined with the nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov/nucleotide/) were used to find similar sequences, type strains and outgroup taxa. ClustalW sequence alignment and phylogenetic analyses were done using the Neighbour Joining on pairwise distance (p-distance) method with 1000 bootstrap replications in the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software (Tamura et al., 2013).

2.3. Results

Two sources of fish were available as raw material for the production of *pindang*. Fresh Longtail tuna (*Thunnus* sp.) caught in local water was transported directly from the fishing port to the processor and frozen Eastern Little tuna (*Euthynnus* sp.) was purchased from local suppliers. The Eastern Little tuna were collected from fishing ports outside Sukabumi regions, such that the fish

processors considered this fish to be “imported” fish. Representative samples of raw, thawed, washed and cooked fish were collected from local processors.

2.3.1. Screening and identification of histamine producing bacteria

Presumptive HPB were identified on modified Niven’s media as purple coloured colonies, with or without halos. In total, 138 typical colonies were obtained from Niven’s media, consisting of 102 mesophilic and 36 psychrotrophic colonies. Qualitative analysis of HPB in HD broth revealed that 117 isolates (84% of the total isolates positive on Niven’s media) converted histidine into histamine after incubation, while the remaining isolates did not and were discarded from further consideration.

Histamine-positive isolates in HD broth were screened for the *hdc* gene. Forty-one isolates, representing 30% of the total isolates, were positive for the *hdc* gene. Among them, 29 isolates (21 mesophilic and 8 psychrotrophic) originated from frozen imported fish. Only 12 HPB-positive isolates were from local fish, which consisted of six mesophilic and six psychrotrophic bacteria.

Identification of the isolates was done using the API® 20E™ biochemical identification system and by sequencing 16s rRNA gene (Table 2-2). However, due to the limitation of API® 20E™ system, not all of the positive isolates were identified. Amongst 19 isolates identified by API, four (21.1 %) gave excellent identification, three (15.8%) gave very good identification, seven (36.8%) gave good identification, three (15.8%) doubtful identification, and two (10.5%) isolates were unidentified.

Table 2-2. Identification of *hdc* gene positive isolates based on 16S rRNA gene sequences and API® 20E, isolated from local fish

Isolates		**16S rDNA-based identification**			**API-based identification**		
		Related sequences	**%ID**	**Genbank accession nb.**	**Significant taxa**	**Identification level**	**%ID**
		Source: Local fish					
Mesophilic	RL8	*Shigella flexneri* ATCC 29903	99	NR_026331.1	NT		
	WL13	*Vibrio alginolyticus* ATCC 17499	99	NR_117895.1	*Vibrio alginolyticus*	Very good	99.8
	CL9	*Enterobacter hormaechei* strain 0992-77	97	NR_042154.1	NT		
	CL10	*Enterobacter* sp.	98	NR_024640.1	NT		
	CL11	*Enterobacter cloacae* ATCC 13047	98	NR_118568.1	NT		
Psychrophilic	WL3_15	*Serratia liquefaciens* ATCC 27592	98	NR_121703.1	NT		
	WL5_15	*Erwinia persicina* NBRC 102418	99	NR_114078.1	NT		
	CL3_15	*Enterobacter* sp.	98	NR_042154.1	NT		
	CL4_15	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	NT		
		Source: Imported fish					
Mesophilic	RI1	*Vibrio parahaemolyticus* ATCC 17802	100	NR_118569.1	*Vibrio parahaemolyticus*	Doubtfull profile	51
	RI2	*Vibrio parahaemolyticus* ATCC 17802	100	NR_118569.1	*Enterobacter cloacae*	Doubtfull profile	92
	RI5	*Vibrio parahaemolyticus* ATCC 17802	100	NR_118569.1	*Eschericia fergusonii*	Good	97
	RI7	*Vibrio parahaemolyticus* ATCC 17802	100	NR_118569.1	*Vibrio parahaemolyticus*	Excellent	100
	TI2	*Providencia rustigianii* DSM 4541	99	NR_042411.1	*Providencia alcalifacien/ rustigianii*	Very good identification to the genus level	84
	TI5	*Pseudomonas putida* ATCC 12633	99	NR_114479.1	Unidentified	Unacceptable profile	
	TI11	*Morganella morganii* ATCC 25830	99	NR_117793.1	*Morganella morganii*	Very good	100
	TI20	*Morganella morganii* subsp. morganii KT	100	NR_102517.1	*Morganella morganii*	Excellent	100

Isolates		16S rDNA-based identification			API-based identification		
		Related sequences	%ID	Genbank accession nb.	Significant taxa	Identification level	%ID
	TI22	*Providencia retgerii* DSM 4542	99	NR_042413.1	*Providencia rettgeri*	Excellent	100
	TI24	*Enterobacter aerogenes* ATCC 13048	98	NR_118556.1	NT		
	TI25	*Hafnia paralvei* ATCC 29927	98	NR_116898.1	NT		
	TI27	*Klebsiella michiganensis* W14	98	NR_118335.1	NT		
	CI3	*Pantoea dispersa* LMG 2603	100	NR_116755.1	*Enterobacter sakazakii*	Good	98
	CI5	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	*Enterobacter cloacae*	Good	95
		Citrobacter freundii ATCC 8090	99	NR_028894.1			
	CI6	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	Unidentified	Unacceptable profile	
		Citrobacter freundii ATCC 8090	99	NR_028894.1			
	CI7	*Pantoea dispersa* DSM 30073	99	NR_116797.1	*Enterobacter sakazakii*	Good	98
	CI8	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	*Enterobacter cloacae*	Good	95
		Citrobacter freundii ATCC 8090	99	NR_028894.1			
	CI9	*Pantoea dispersa* DSM 30073	99	NR_116797.1	*Enterobacter sakazakii*	Doubtfull profile	95
	CI11	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	*Enterobacter cloacae*	Good	95
		Citrobacter freundii ATCC 8090	99	NR_028894.1			
	CI22	*Enterobacter cloacae* ATCC 13047	98	NR_118568.1	NT		
Psychrophilic	CI1_15	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	*Enterobacter cloacae*	Good	95
		Citrobacter freundii ATCC 8090	99	NR_028894.1			
	CI2_15	*Proteus mirabilis* ATCC 29906	99	NR_114419.1	*Proteus mirabilis*	Excellent	100

Isolates		16S rDNA-based identification			API-based identification		
		Related sequences	%ID	Genbank accession nb.	Significant taxa	Identification level	%ID
	CI3_15	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	NT		
	CI5_15	*Enterobacter cloacae* ATCC 13047	99	NR_118568.1	NT		

Note:NT : Not tested, C = Cooked, R = Raw, T = Thawed, W = Washed

Analysis of 16S rRNA gene sequencing confirmed all isolates had ≥97% sequence similarity to sequences in GenBank. Isolates RI1, RI2, RI5 and RI7 had 100% sequence similarity to *Vibrio parahaemolyticus* strain ATCC 17802 (NR 118569.1), while isolate TI20 and isolate CI3 had 100% homology with *Morganella morganii* subsp. *morganii* (NR 102517.1) and *Pantoea dispersa* strain LMG 2603 (NR 116755.1), respectively. In total, fourteen different genera were identified, *i.e*. *Citrobacter, Enterobacter, Escherichia, Erwinia, Hafnia, Klebsiella*, *Morganella, Pantoea, Proteus, Providencia, Pseudomonas, Serratia, Shigella* and *Vibrio*. Genera with similar percentage of sequence similarity were assigned in the same group, such as *Escherichia-Shigella* and in some cases *Citrobacter-Enterobacter*.

2.3.2. Histamine quantitative analysis

Further confirmation of *hdc* gene positive isolates' ability to produce histamine in broth media showed that mesophilic isolates in histidine broth produced considerably higher amounts of histamine, ranging from 2.5 to more than 4,000 ppm (Table 2-3). The mesophilic isolates tested achieved total viable counts (TVC) of 8.94 ± 0.54 log CFU/ml on average in stationary phase. The maximum levels of histamine produced by the psychrotrophic isolates was 4.5 ppm, with an average TVC of 8.67 ± 0.12 log CFU/ml. Due to the limitation of the availability of the quantitative histamine analysis kit, not every confirmed isolate was tested for its ability to produce histamine in histidine broth.

The reference isolates, *M. morganii* (32) and *M. psychrotolerans* (FE 3_12) were able to produce 4,542 ppm and 3,130 ppm histamine, respectively, at the corresponding incubation conditions. At the end of incubation, *M. morganii* (32) and *M. psychrotolerant* (FE 3_12) reached TVC of 9.4 and 8.7 log CFU/ml, respectively.

The histamine levels measured in the fish from local waters were significantly higher ($P<0.01$) than in imported fish. Raw and cooked-local fish contained 12.5 and 10.7 ppm, respectively, while raw and cooked-imported fish were 2 and 1.08 ppm, respectively.

Table 2-3. Histamine level of the tested isolates

	Source	Isolates	Histamine level (ppm)	16S rRNA-based identification
Mesophilic	Local fish	RL8	19.8	*Shigella flexneri* ATCC 29903
		WL17*	0.89	
		CL9	2.5	*Enterobacter* sp.
		CL10	2.5	*Enterobacter* sp.
		CL11	30.23	*Enterobacter cloacae* ATCC 13047
	Imported fish	TI24	4,314	*Enterobacter aerogenes* ATCC 13048
		TI25	84.8	*Hafnia paralvei* ATCC 29927
		TI27	18.35	*Klebsiella* sp
		CI22	17.72	*Enterobacter cloacae* ATCC 13047
Psychrophilic	Local fish	WL3_15	3.59	*Serratia liquefaciens* ATCC 27592
		WL4_15*	0.21	
		WL5_15	1.92	*Erwinia* sp.
		CL2_15*	0.42	
		CL3_15	4.5	*Enterobacter* sp.
		CL4_15	3.33	*Enterobacter cloacae* ATCC 13047
	Imported fish	CI3_15	1.38	*Enterobacter cloacae* ATCC 13047
		CI5_15	3	*Enterobacter cloacae* ATCC 13047

*Note: *Due to background colour given by the broth media, isolates produce histamine less than 1 ppm were considered as non-histamine producer and not identified. C = Cooked, R = Raw, T = Thawed, W = Washed*

2.4. Discussion

Two sources of fish were available as raw material for *pindang*: 1) fresh tuna caught from local waters and transported directly from the fishing port to the processor, 2) frozen tuna purchased from local suppliers. The consumption of tuna, and other species in Scombroidae family, including mackerels and bonitos, is the most prevalent source of HFP, as this family has naturally high levels of histidine in muscle tissue (FAO & WHO, 2012; Kim et al., 2004; Knope et al., 2014; Lavon et al., 2008; Lehane & Olley, 2000; Taylor et al., 1989).

Comprehensive data on the risk of HFP in Indonesia, comprising the fish sources, the characteristics of associated foods and processing conditions, and the number of victims are currently being developed. However, data on the status of histamine contamination in fish products from Indonesia that are exported to some European countries can be retrieved from EU RASFF portal (https://webgate.ec.europa.eu/rasff-window/portal/). In the period from 1998 to May 2017, 59 out of 240 total alerts (24.6%) issued by eleven countries were for the presence of histamine in fresh, frozen, canned and vacuum-packed tuna at levels higher than the maximum allowed by the European Commission (European Commission, 2005).

In the current study, different species of HPB were isolated from raw, thawed, washed and cooked *pindang*. Isolates from genera *Escherichia/Shigella* and *Vibrio* were found in raw and washed-local fish whereas *Vibrio* spp. were also isolated from raw-imported fish. *E. coli* was categorized as non-indigenous bacteria in seafood product (Huss, 1994) and has been known as an indicator of faecal contamination in the tropical marine environment (Costa, 2013; Huss, 1994; Thampuran et al., 2005). *V. parahaemolyticus* and other vibrios are indigenous marine bacteria found in a wide variety of fish and shellfish, especially those caught from tropical waters including Indonesia (Huss, 1994; Molitoris et al., 1985). The presence of *Vibrio* spp. in the washed but not in the raw-local fish is possibly due to the use of contaminated seawater for washing.

Vibrio spp. and *Escherichia/Shigella* were not isolated from the final salted-boiled product made either with local or imported fish. Both microorganisms readily killed by normal cooking (Lake et al., 2003; Miles et al., 1997) *e.g.*, at temperatures of at least 63°C (US FDA, 2012), therefore they would be expected to be eliminated during the processing of *pindang*, where fish are steamed for approximately two hours.

High HPB, such as *Enterobacter* sp., *Klebsiella* sp., *Morganella* sp. and *Providencia* sp. were isolated from the thawed-imported fish. Amongst the *Enterobacteriaceae*, *Hafnia alvei* and *Klebsiella pneumonia* are the most common species associated with HFP cases (Lehane & Olley, 2000; Taylor & Speckhard, 1983). The natural presence of *Morganella, Klebsiella* and *Hafnia* on the surface of live fish, in fish gills and intestines and the ability of these microorganisms to multiply and spread to different parts of fish during handling, and when spoilage begins increases the possibility of histamine formation in fish (Lehane & Olley, 2000; Sumner et al., 2004). Furthermore, the microflora of fish caught from polluted waters and spoiled at a warm temperature (25°C) are more likely to be dominated by the *Enterobacteriaceae* group of bacteria (Visciano et al., 2012). During processing of imported fish, raw-frozen fish was thawed or defrosted under uncontrolled temperature and time conditions, providing favourable conditions for bacterial growth, histamine production and spoilage.

High HPB were not found in the washed-local fish for which no thawing stage was involved in the processing. Therefore the current thawing or defrosting practice, where the temperature was not well-maintained, may have a profound effect on the growth of bacteria in pre-cooked fish, especially the pre-existing HPB. In addition, no gutting was involved in the fish preparation, thus increasing the opportunity for gut microbiota to grow and contaminate other parts of the fish.

Insufficient packaging of the final product can allow post-processing contamination. Prior to cooking, fish bellies are wrapped with paper to avoid rupture during cooking (Murtini et al., 2013). The paper remains on the cooked fish and is used as packaging when the fish are sold in the market or by the fish peddlers. The presence of *Enterobacteriaceae* members such as *Enterobacter* sp.,

Pantoea sp., *Proteus* sp. and *Citrobacter* sp. that were isolated from the cooked products of local and imported fish is most likely as a result of post-processing contamination, as they would not have survived the cooking process. This family of bacteria is an indicator of poor hygienic and sanitation conditions in food processing plants (Baylis et al., 2011). A previous study of salted-boiled tuna (*Auxis rochei*) from Indonesia also identified *Proteus vulgaris, H. alvei, M. morganii, Enterobacter aerogenes, Klebsiella oxytoca,* and *K. pneumonia* from the cooked product (Fatuni, 2014), showing that post-processing contamination of the finished product is common.

Histamine analysis of HPB isolates in broth media showed that isolate TI24 produced ≥4,000 ppm of histamine in broth media at 30°C, whilst isolate TI25 produced 84.8 ppm. Isolate TI24 and TI25 has 98% similarity with *E. aerogenes* strain ATCC 13048 and *Hafnia paralvei* strain ATCC 29927, respectively. *E. aerogenes*, *H. alvei*, *K. oxytoca*, *K. pneumonia* and *Klebsiella ornithinolytica* isolated from different types of fish such as tuna, bonito, sardine and mackerel have previously been shown to be high HPB (produce ≥ 1,000 ppm) in broth media (Jiang et al., 2013; Kanki et al., 2002; Torido et al., 2014; Tsai et al., 2004). *Enterobacter* spp. isolated from fermented mackerel ('*peda*') from Indonesia produced histamine at 305.49 mg/100 ml of broth media which was higher than *M. morganii* (92.35 mg/100 ml) incubated in the same conditions (Indriati et al., 2006; Mangunwardoyo et al., 2007).

HPB isolated at 17°C, such as *Citrobacter* sp., *Serratia* sp., *Enterobacter* sp*., Erwinia* sp. and *Proteus* sp., indicated the risk of histamine formation is still present in the product when it is stored at relatively low temperatures. Moreover, isolation of *Citrobacter* sp. or *Enterobacter* sp. and *Proteus* sp. from the cooked product showed the presence of post-processing contamination, as mentioned previously. Although they produce lower levels of histamine than the mesophilic isolates, the ability of these genera to survive and proliferate in the finished product, shows the risk of histamine formation in the production process of *pindang* is still present.

Although HPB were isolated from the finished product, histamine analysis of *pindang* showed an acceptable level of histamine in both the raw and cooked fish product, which is ≤ 100 mg/kg for salted-boiled fish or salted fish (SNI 2717.1:2009) . However, the existence of HPB in the fish during the processing and the current processing practice where temperature and hygienic condition can be inadequate, might activate bacterial growth and trigger histamine accumulation in the product before it reaches the consumers. Based on the current processing practice, the expected shelf-life of this product is two to three weeks when stored at room temperature and packed properly (Ariyani et al., 2010). Histamine formation in tuna stored at low temperature is slight, and not a significant health risk, unless the fish are exposed to significant time delay during the processing (López-Sabater et al., 1996).

2.5. Conclusion

Low and high HPB were found in *pindang* from Indonesia. High HPB, such as *Enterobacter* sp., *Klebsiella* sp., *Morganella morganii* and *Providencia* were isolated from the thawed-imported fish. High HPB were not found in the washed-local fish when no thawing stage was involved. The other HPB isolated from the fish were *Citrobacter* sp., *Erwinia* sp., *Escherichia* sp., *Hafnia* sp., *Pantoea* sp., *Proteus* sp., *Providencia* sp., *Pseudomonas* sp. and *Serratia* sp.

The current processing practices to produce *pindang*, primarily fish thawing or defrosting, can allow for the growth of HPB, including high histamine producers in the fish, and production of toxic levels of histamine in the product. These bacteria can be eliminated during processing by steaming, however heat-resistant histamine that can form during the processing could persist in the final product. The presence of *Enterobacteriaceae* family in the cooked product suggested post-processing contamination might occur due to imperfect packaging of the final product and also contribute to histamine levels.

Chapter 3. Growth and Histamine Formation by *Enterobacter aerogenes* Isolated from Indonesian *Pindang*

3.1. Introduction

Seafood consumption is the most prominent cause of HFP (Huss & Gram, 2004; Taylor, 1986), an adverse health condition with symptoms similar to food allergy (Hungerford, 2010). Different type of fish and fish products that have caused HFP were reviewed in Chapter 1 (Section 1.2.1). Certain tolerable levels of histamine in fish products have been decreed by authorities from different countries. The Australia New Zealand Food Standard (ANZFS) code currently establishes the maximum levels of histamine as a natural toxicant in fish and fish products at 200 mg/kg (FSANZ, 2017). The Canadian Food Safety and Inspection authority allows histamine levels up to 200 mg/kg for enzyme ripened products such as anchovies, anchovy paste and fish sauce; and 100 mg/kg for all other fish products including canned or fresh or frozen tuna, mackerel and mahi-mahi (CFIA, 2017). The European Commission also specifies safe levels of histamine in fisheries products from fish species associated with higher amounts of histidine (Reg. EC 2073/2005). A three-class sampling plan is suggested by the EC, where amongst nine sets of samples, no more than two samples may exceed 100 mg/kg histamine ('m'), and no sample may exceed 200 mg/kg histamine ('M') (EC, 2005). Amongst other authorities, the US requires the lowest allowable limit of histamine as a maximum of 50 mg/kg of histamine for seafood to be categorized as proper for consumption, while products with 500 mg/kg of histamine are considered as "unsafe" for consumption and might cause health problem for consumers (US FDA, 2011; US FDA, 2005). FAO & WHO determined that the histamine concentration in fish should not exceed 200 mg/kg (FAO & WHO, 2012). This value was obtained based on fish consumption data from several countries (European countries, Thailand, Japan, UK, USA and New Zealand), and based on an experts consultation. The calculation was made by assuming the maximum serving size in these countries is 250 g and the tolerable hazard level of histamine is 50 mg/kg.

Indonesian *pindang* is traditionally made from Scombroid fish such as tuna and mackerel. The production of *pindang* is the second biggest product amongst other traditional fish products in Indonesia with 100,000 tonnes produced in 2010 (Directorate General of Capture Fisheries, 2011). The Indonesian National Standard (SNI 2717.1:2009, SNI 2729:2013) regulates the maximum limit of histamine for *pindang* and raw material used in the processing of *pindang*, at 100 mg/kg (BSN, 2009b; BSN, 2013).

Results presented in Chapter 2 of this thesis showed that the use of fresh fish as a raw material in the processing of *pindang* does not support the growth of high histamine producing bacteria. However, high levels of HPB were found in *pindang* made from frozen (imported) fish, where thawing was involved in the processing. *Enterobacter* sp., *Klebsiella* sp., *Morganella* sp. and *Providencia* sp. were isolated from the imported fish. From Chapter 2, it was shown that *Enterobacter aerogenes* produced a significant level of histamine (4,314 µg/ml) when incubated at 30°C in histidine-supplemented broth media.

Control of histamine formation during the processing of *pindang* is necessary to avoid high accumulation of this compound in the final product. Mathematical approaches have been used to model the growth of bacteria at specific conditions as well as predict their metabolic activities. Knowing how the bacteria behave during the conditions of processing of *pindang* could lead to the introduction of intervention strategies which can reduce or prevent histamine formation and accumulation in the fish product.

This study aims to model and predict growth and histamine formation by *E. aerogenes* isolated from *pindang*, at different temperatures. The effect of salt and thawing conditions, which mimics the real processing of the fish, on bacterial growth and histamine formation was also investigated.

3.2. Materials and methods

3.2.1. Experimental conditions: growth rate and histamine production

Enterobacter aerogenes TI24 was isolated from *pindang* from Indonesia (see Chapter 2). Prior to the experiment, the isolate was cultured in tryptone soy broth (TSB) (Oxoid, UK) at 30°C for 24 h. The overnight culture was diluted and transferred into an Erlenmeyer flask containing 45 ml of histidine broth (1% bacteriological peptone (Oxoid, UK), 0.3% yeast extract (Oxoid, UK), 1.5% sodium chloride (Merck, USA) and 0.5% of L-histidine (Sigma, USA)), to obtain a final cell density of 10^3 – 10^4 CFU/ml. The flasks were incubated with agitation in a shaking water bath (Ratek, AU) at 30°; 20°; 15° and 10°C for 24 h, 36 h, 12 d and 16 d, respectively. Bacterial growth and histamine concentration were determined at regular intervals during the incubation (as described in Section 2.3. and 2.4.). Triplicate experiments were done to determine the bacterial growth curves.

To study the effect of salt on bacterial growth and histamine formation, the isolate was grown in histidine broth containing 6%, 10% and 20% NaCl and incubated at 10°; 15°; 20° and 30°C. NaCl at 6 and 10% represent the amount of salt added by the fish processors during *pindang* processing, while 20% of NaCl is the recommended amount of salt to be added during the processing (SNI 2717.3:2009).

3.2.2. Thawing experiment

A thawing experiment was designed to mimic the thawing step in *pindang* processing. Two initial densities of *E. aerogenes* culture in broth were used in these experiments. The density of group A and B were approximately 10^7 and 10^4 CFU/ml, respectively. The culture was frozen at -20°C for 3 d and thawed at 4°, 18° and 25°C for 4 h. During thawing, bacterial growth and histamine formation were measured at different intervals.

3.2.3. Bacterial enumeration

Serial dilutions of cell cultures were made in phosphate buffered saline (PBS) (0.24 g potassium phosphate monobasic (KH_2PO_4) (Merck, USA), 1.44 g sodium phosphate dibasic ($Na_2HPO_4.7H_2O$) (Merck, USA), 8 g sodium chloride (NaCl) (Merck, USA) were dissolved in 1 l of distilled water) with pH adjusted at 7.4. Aliquots (100 µl) of the diluted cell suspensions were spread-plated onto tryptone soya agar (Oxoid CM0129, UK, with 1.5% agar), and incubated at 30°C for 24 h. Colonies were counted and expressed as log CFU/ml.

3.2.4. Histamine extraction

The bacterial culture was centrifuged at 10,000 x*g* for 2 min. A 1 ml aliquot of the supernatant was collected and filtered through a 0.22 µm cellulose acetate membrane filter. Serial dilutions were made using MilliQ water and 100 µl of 10 µg/ml of histamine.2HCl (α, α, β, β-D4, 98%) (Novachem, AU) was added to the sample as an internal control standard.

3.2.5. Histamine quantification

A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was coupled with a Waters Acquity UPLC PFP column (2.1 mm × 100 mm × 1.7 µm) for analysis. The mobile phase consisted of two solvents: 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). The UPLC program was 100% A to 60% A:40% B at 3.0 min, which was held for 0.5 min, and this was followed by immediate re-equilibration to starting conditions for 3 min. The flow rate was 0.20 ml/min. The columns were held at ambient temperature, and the sample compartment at 6°C. Injection volume was 10 uL. Approximate retention time for Histamine was 2.9 min.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.8 kV, and multiple reactions monitoring (MRM) was used to detect all analytes. For histamine, the MRM transitions: precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 95.0 $[M+H]^+$ was used for quantitation with a dwell time of 22 msec, cone voltage was 21 V and collision energy 12 V; precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 68.0 $[M+H]^+$ was used for confirmation, cone voltage

21 V and collision energy 20 V. For D4-Histamine the MRM transitions: precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 99.0 $[M+H]^+$ was used for quantitation; precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 72.0 $[M+H]^+$ was used for confirmation, with the same cone voltages and collision energies. The ion source temperature was 130°C, the desolvation gas was N_2 at 950 l/h, the cone gas flow was 100 l/h, and the desolvation temperature was 450°C. Data were processed using MassLynx software (Waters Corp, 2018).

3.2.6. Modelling the growth parameter and histamine production of *E. aerogenes*

The maximum growth rates (μ_{max}), lag time (λ) and maximum population density (N_{max}) of the isolate at each temperature (See Section 3.2.1) were determined by fitting the experimental data (log count) to the Baranyi and Roberts model (Equation 3-1 – Equation 3-3) (Baranyi & Roberts, 1994) using the desktop version of DMFit Version 3.5 (www.combase.cc).

$$ln\big(N(t)\big) = ln(N_0) + \mu_{max}A(t) - ln[1 + \frac{e^{\mu_{max}A(t)}-1}{e^{(N_{max}-N_0)}}] \quad \text{(Equation 3-1)}$$

$$A(t) = t + \frac{1}{\mu_{max}} ln(\frac{e^{(-\mu_{max}t)}+q_0}{1+q_0}) \quad \text{(Equation 3-2)}$$

$$\lambda = \frac{ln(1+\frac{1}{q_0})}{\mu_{max}} \quad \text{(Equation 3-3)}$$

where:

$ln\big(N(t)\big)$ = log of cell concentration at time *t* (h), in CFU/ml; $ln(N_0)$ = log of initial cell concentration, in CFU/ml; $ln(N_{max})$ = log of maximum cell concentration; μ_{max} = maximum growth rate (h^{-1}); and λ = lag time (h).

The kinetic data obtained from this study were deposited in ComBase (www.combase.cc) following publication of the thesis under the source name "Rachmawati".

Secondary models to describe the effects of temperature on the maximum growth rate (μ_{max}) were determined using multiple linear regression (Equation 3-4) and an extended square root model (Equation 3-5) (McMeekin et al., 1987).

$$\mu_{max} = a + bT + cT^2 + dS + eS^2 + fTS \quad \text{(Equation 3-4)}$$

where:

μ_{max} = maximum growth rate, T = temperature (°C), S = salt concentration (%)

$$\sqrt{\mu} = b\ (T - T_{min})\sqrt{(a_w - a_{wmin})} \quad \text{(Equation 3-5)}$$

where:

μ_{max} = maximum growth rate, T_{min} = theoretical minimum temperature that supports the growth (°C), a_w = water activity (%), a_w = theoretical minimum a_w that supports the growth.

a_w was calculated using Equation 3-6 and Equation 3-7, as described by Ross and Dalgaard (2004)

$$a_w = 1 - 0.0052471 * \%WPS - 0.00012206 * \%WPS^2 \quad \text{(Equation 3-6)}$$

$$\%WPS = \%NaCl(w/v) * 100/(100 - \%dry\ matter + \%NaCl\ (w/v)) \quad \text{(Equation 3-7)}$$

Since the formation of histamine depends on the number of the bacterial cells (Rodriguez-Jerez et al., 1994), the rate of histamine formation can be correlated with the bacterial absolute growth rate, using a yield factor (Equation 3-8, Equation 3-9) (Emborg & Dalgaard, 2008a, 2008b; James et al., 2013; Jorgensen et al., 2000). Using the observed and modelled histamine concentrations, the time required by *E. aerogenes* to produce 100 µg/ml of histamine in broth was estimated.

$$His(t) = His(t_0) + Y_{His/CFU} * \left(CFU(t) - CFU(t_0)\right) * 1000 \quad \text{(Equation 3-8)}$$

$$pY_{His/CFU} = -log\left(\frac{His(final) - His(t_0)}{CFU(final) - CFU(t_0)}\right) \quad \text{(Equation 3-9)}$$

where:

$pY_{His/CFU}$ is the constant yield factor; $CFU\ (t_0)$, $CFU(final)$ and $CFU(t)$, are colony forming units per ml at initial, final and time (t), respectively; $His(t_0)$, $His(final)$ and $His\ (t)$ are histamine concentration in µg/ml at initial, final and time (t), respectively.

3.2.7. Statistical analysis

The growth parameters of *E. aerogenes* at different temperatures and salt concentrations were analysed using analysis of variance (ANOVA) in SigmaPlot Version 12.5. Significant difference was defined at P<0.001.

The relative (percentage) deviations between predicted and observed times to produce 100 µg/ml of histamine was calculated as: $\frac{(\text{predictive value} - \text{observed value}) \text{x} 100}{\text{observed value}}$ (Emborg & Dalgaard, 2008a).

To evaluate performance of the model, the Root Mean Square Error (RMSE) and the coefficient of determination (R^2) from the secondary models were compared. Calculations were done using Microsoft Excel® software.

3.3. Results

3.3.1. Bacterial growth and histamine formation

The average initial counts of *E. aerogenes* was 3.6 ± 0.54 log CFU/ml. *E. aerogenes* was able to grow at all incubation temperatures when no additional salt was added. However, at the higher salt concentrations (6 and 10% NaCl), the bacterial growth depended on temperature, while at 20% of NaCl, growth was not observed at any incubation temperature (Figure 3-1).

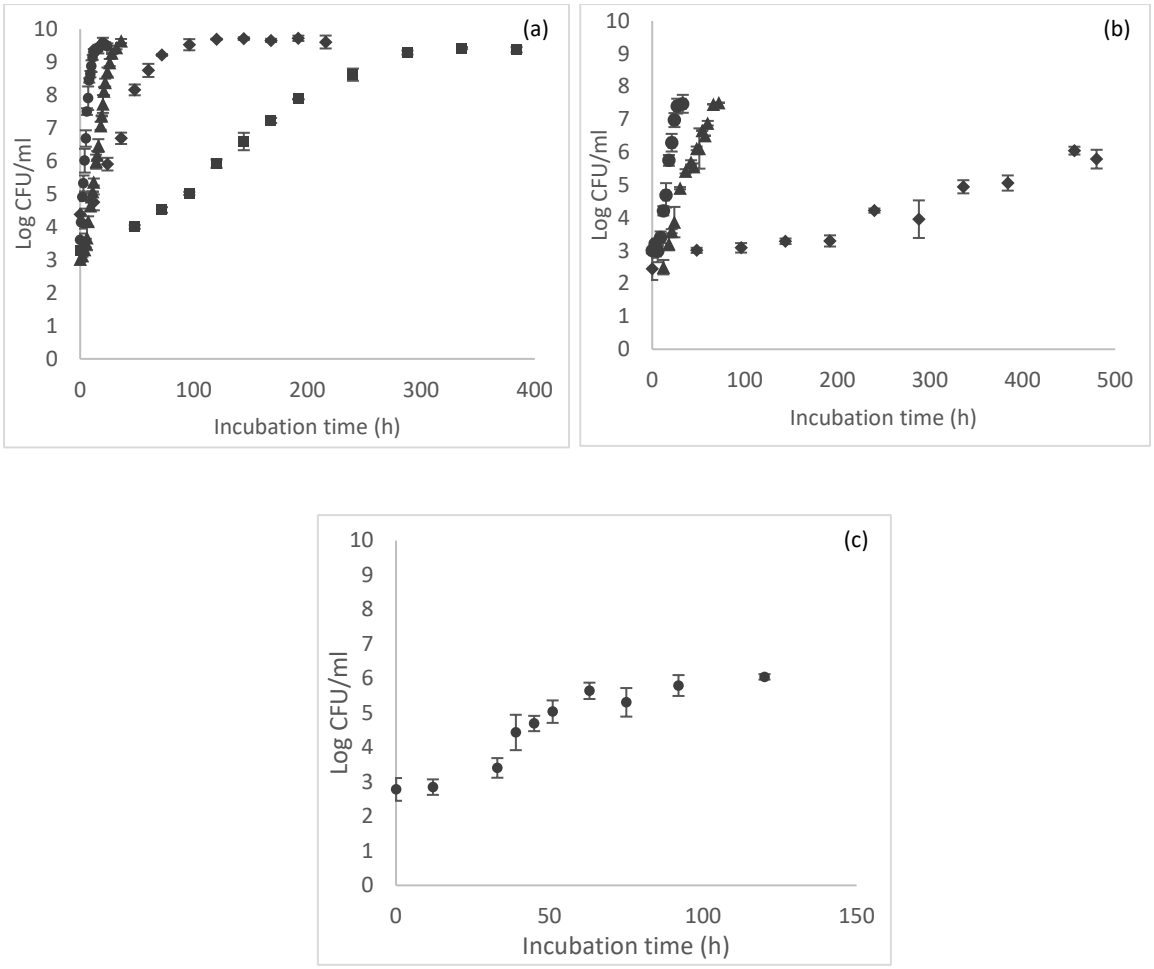

Figure 3-1. Growth curves of *E. aerogenes* observed at (a) basal concentration of NaCl (1.5%); (b) 6% NaCl; and (c) 10% NaCl in histidine broth. Incubation was performed at (●) 30°C, (▲) 20°C, (◆) 15°C and (■) 10°C.

Although the isolate survived and grew at higher salt concentration (6% and 10%), the histamine production was markedly affected by salt concentration (Figure 3-2). More than 200 µg/ml of histamine was produced at all temperature when no additional salt was added to the broth media (*n.b.*, the basal media contains 1.5% NaCl). The highest concentration of histamine (6,264 µg/ml) was produced at 30°C, during the stationary phase. At 10, 15 and 20°C incubation, the highest concentrations of histamine observed were 3,929 µg/ml, 4,276 µg/ml and 3,999 µg/ml, respectively. These concentrations were observed during the stationary phase of growth of *E. aerogenes*. When 6% of NaCl was added into the growth media, the highest level of histamine (363 µg/ml) was found at 30°C incubation. This level was observed between the late exponential and

early stationary phase of the isolate's growth. Furthermore, a negligible amount of histamine (<5 µg/ml) was found at lower incubation temperatures and higher salt concentration.

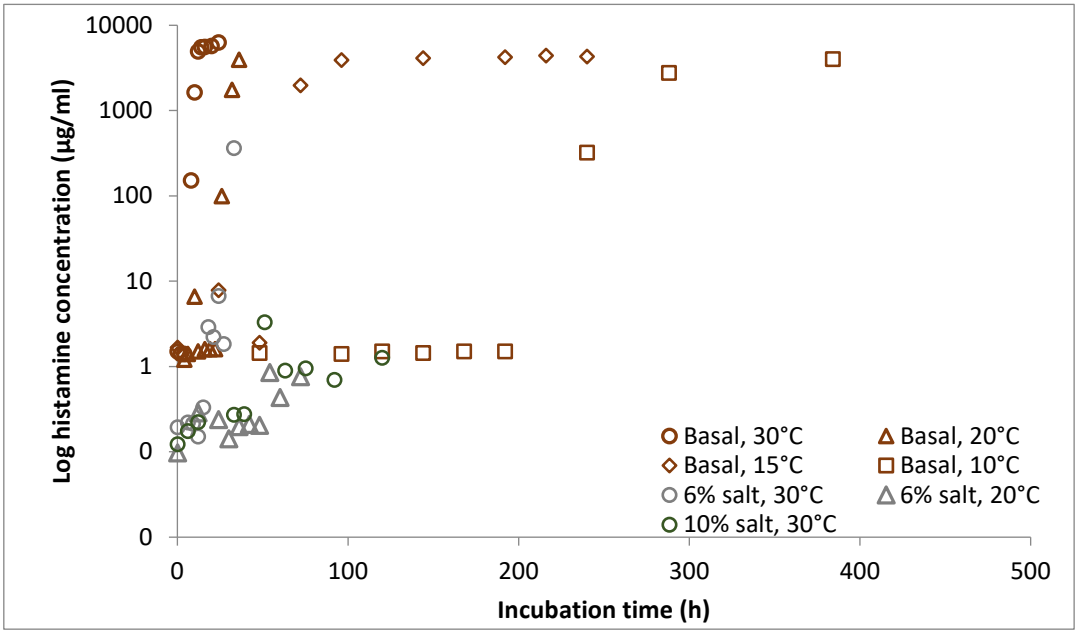

Figure 3-2. Histamine production of *E. aerogenes* at different temperatures and salt concentrations

3.3.2. Modelling the histamine formation of *E. aerogenes*

Relative histamine yield per cell bacteria observed during the growth is shown in Figure 3-3. The amount of histamine produced per cell increased during the exponential phase of bacteria, while slight decrease followed by constant values were observed at late exponential and stationary phase. Prior to late exponential phase, a lag of histamine production was observed.

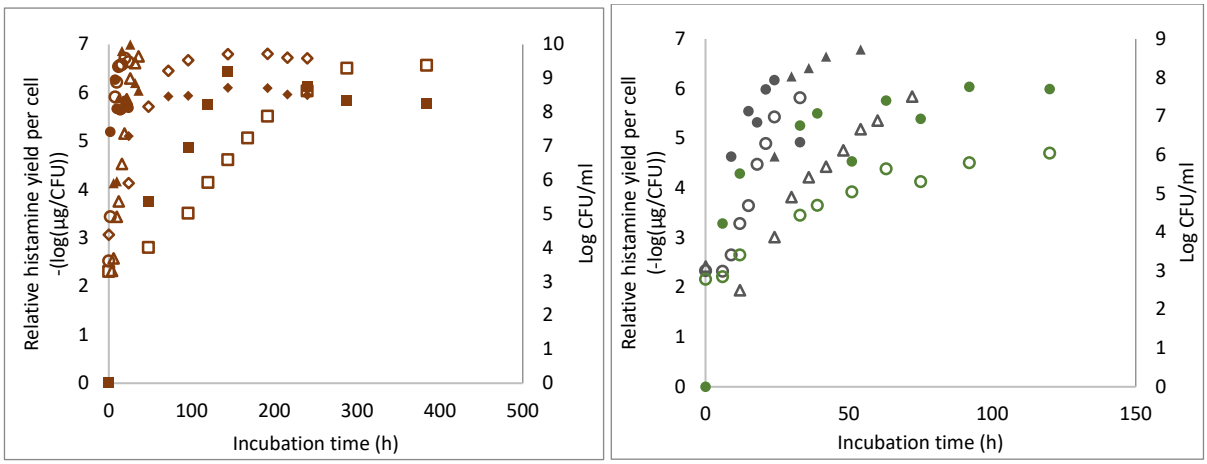

Figure 3-3. Histamine production per cell (closed symbols) by *E. aerogenes* as observed during the growth (open symbols) at 10 (□), 15 (◇), 20 (△) and 30°C (○) with 1.5 (red), 6 (grey) and 10% (green) of salt concentrations.

From equation 3-9, the yield factors of histamine at different incubation conditions are presented in Table 3-1. There were no significant differences in the histamine yield factors amongst treatments.

Table 3-1. Yield factor ($pY_{His/CFU}$) of histamine in broth during incubation

Salt (%)	Temperature (°C)	*pYhis/CFU* (-log(µg/CFU))
1.5	10	5.919 ± 1.15
	15	6.233 ± 1.08
	20	6.314 ± 1.52
	30	5.696 ± 0.29
6	20	6.609 ± 0.93
	30	5.427 ± 0.6
10	30	5.115 ± 0.91

Using the obtained yield factors, the predicted histamine produced by *E. aerogenes* can be estimated (Figure 3-4).

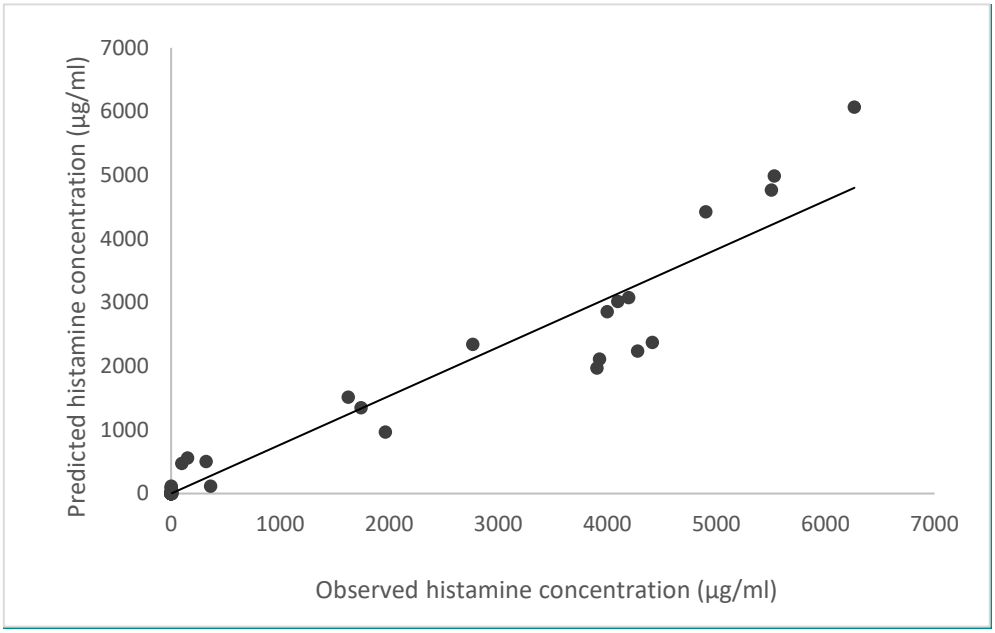

Figure 3-4. Predicted and observed histamine concentration produced by *E. aerogenes* in broth (R^2=0.939)

The time required by *E. aerogenes* to produce 100 µg/ml of histamine was estimated using Equation 3-10. A squared-root model (Ratkowsky et al., 1982) was used to describe the effect of temperature, and combined with additional, novel, term for the effect of salt concentration. The general form of the model is:

$$t_{100} = \frac{a \times (\frac{S}{Smax - S})}{(T - Tmin)^2}$$ Equation 3-10

where:

a is a constant fitting parameter; S_{max} and T_{min} are values to be fitted representing the upper limits of NaCl for histamine production and lower limit of temperature for histamine production, respectively; S = salt concentration (%) and T = temperature (°C) are the independent variables imposed.

$$t_{100} = \frac{23540x(\frac{S}{13.2-S})}{(T-6.1)^2}$$ Equation 3-11

The constant and fitted parameter values of this model were calculated using Solver in Microsoft Excel® (Microsoft Corp.) by minimising the sum of square differences between the predicted and observed values of time needed to reach 100 mg/l of histamine. Because the t_{100} values range so widely (nearly two orders of magnitude), the error to be minimised was sum of (log (observed) - log (fitted)). The observed and predicted times to produce 100 µg/ml histamine are presented in Figure 3-5.

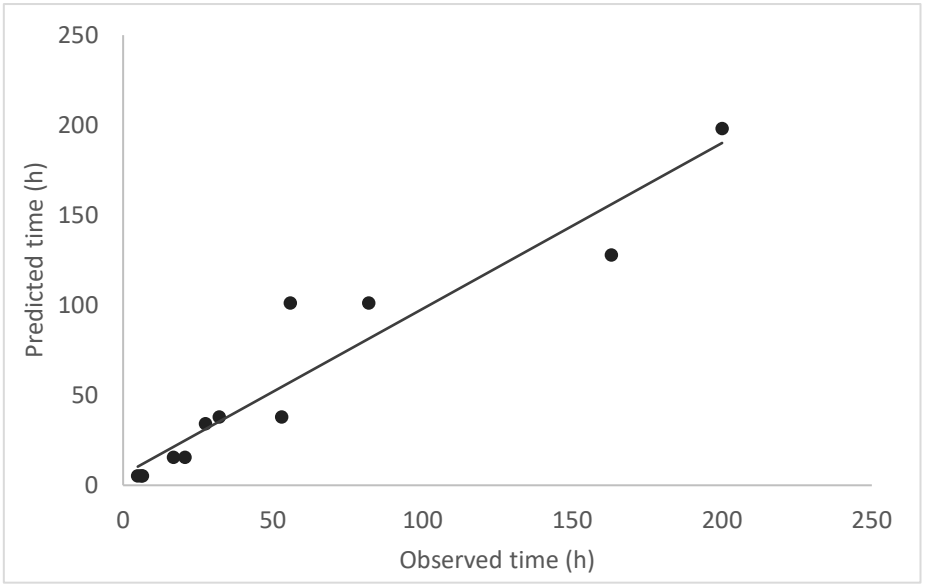

Figure 3-5. Predicted and observed time required by *E. aerogenes* to produce 100 µg/ml histamine in broth (R^2=0.9111)

3.3.3. Primary and secondary models for *E. aerogenes* growth kinetics

The average estimated growth parameters, *i.e.* lag time (*λ*), growth rate (μ_{max}) and maximum population density (N_{max}), from three replicates are presented in Table 3-2. λ was significantly (P<0.001) affected by salt and temperature. A longer λ was observed at higher salt concentration and lower temperature. The longest lag phase (121.9 h) was observed at 15°C with 6% of salt. μ_{max} and N_{max} were significantly influenced by temperature and salt concentration, respectively. The highest N_{max} (9.5 log CFU/ml) was reached at the basal media salt concentration (1.5%). The

isolate grew faster at higher temperatures, regardless of the salt concentration. The average R^2 of the fitted growth rate at 1.5%, 6% and 10% of salt were 0.99, 0.95 and 0.92, respectively.

Table 3-2. Growth parameters of *E. aerogenes*

Salt (%)	Temperature (°C)	λ (h)	μ_{max} (h^{-1})	N_{max} (CFU/ml)
1.5 (basal media)	10	30.98 ± 0.1	0.0272 ± 0.002[a]	9.4 ± 0.1[a]
	15	6.69 ± 0.3	0.0845 ± 0.05[b]	9.6 ± 0.1[a]
	20	3.85 ± 0.2	0.2974 ± 0.004[c]	9.5 ± 0.05[a]
	30	-	0.6075 ± 0.02[d]	9.4 ± 0.03[a]
6%	10*	-	-	-
	15	121.9	0.0074 ± 0.002[a]	-
	20	11.99	0.0844 ± 0.01[b]	7.7[b]
	30	8.61 ± 1.1	0.2746 ± 0.01[c]	7.5 ± 0.3[b]
10%	10*	-	-	-
	15*	-	-	-
	20*	-	-	-
	30	26.2	0.0542 ± 0.03[b]	5.9 ± 0.1[c]

Different letters denote significant differences (P<0.05)
**No growth was observed*

A multiple linear regression showing the relationship between growth rate and the environmental parameters (temperature and salt concentration) is presented in Figure 3-6 and described in Equation 3-12. The R^2 and RMSE of the model were 0.9855 and 0.02305, respectively.

$$\mu_{max} = -0.17754 + 0.017884T + 0.000427T^2 + 0.0063S + 0.002217S^2 - 0.00324TS$$

(Equation 3-12)

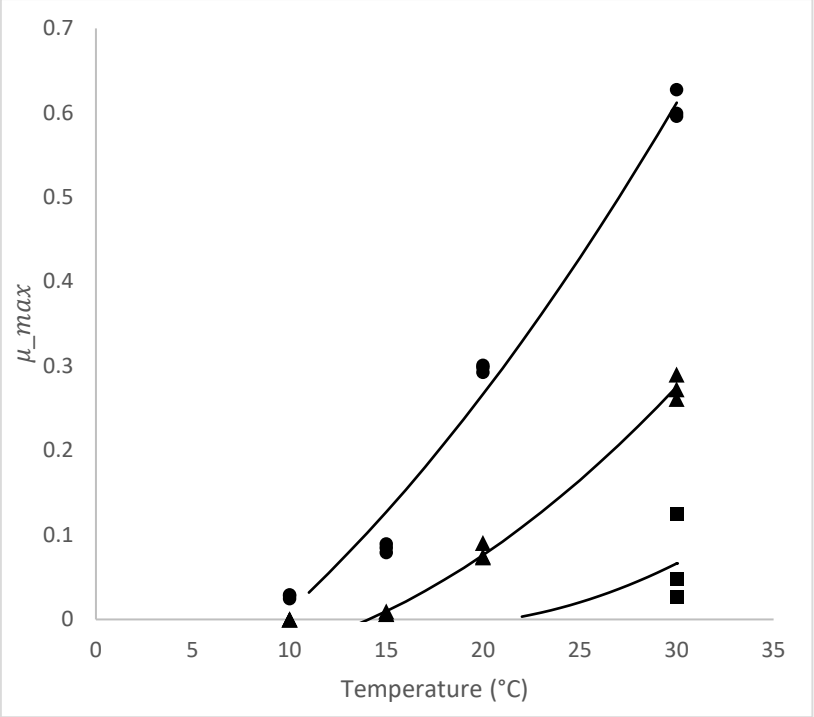

Figure 3-6. Plotted growth rate (μ) values against temperature at 1.5% (●), 6% (▲) and 10% (■) of salt (the multiple linear regression)

In this study, the minimum temperature that supported the growth of *E. aerogenes* (T_{min}) was determined at 4°C, as also suggested from a study in sailfish and milkfish (Tsai et al., 2005a) and the minimum a_w value used was 0.95 (Jay et al., 2005). Using these reference values, Equation 3-13 was obtained from the extended Ratkowsky model. The fitted μ values are shown in Figure 3-7. The R^2 and RMSE of the model were 0.9597 and 0.0415, respectively.

$$\mu_{max} = 0.0170(a_w - 0.9399)(T - 3.9441)^2 \quad \text{(Equation 3-13)}$$

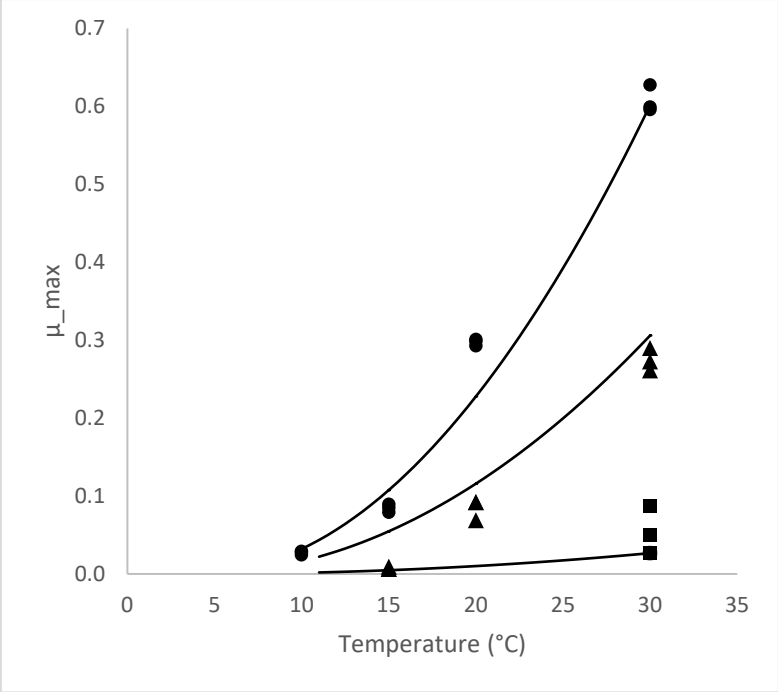

Figure 3-7. Plotted growth rate (μ) values against temperature at 1.5% (●), 6% (▲) and 10% (■) of salt (the extended Ratkowsky model)

3.3.4. The effect of thawing on bacterial growth and histamine formation by *E. aerogenes*

Freezing reduced the number of *E. aerogenes* in broth. A two to three log reduction was observed in the low initial count group (B) after freezing while for the high initial count group (A) a lower log reduction (0.5 - 2 logs) was observed. During thawing at 4°, 18° and 25°C for 4 h, the bacterial counts for both groups remained at the same levels (Figure 3-8).

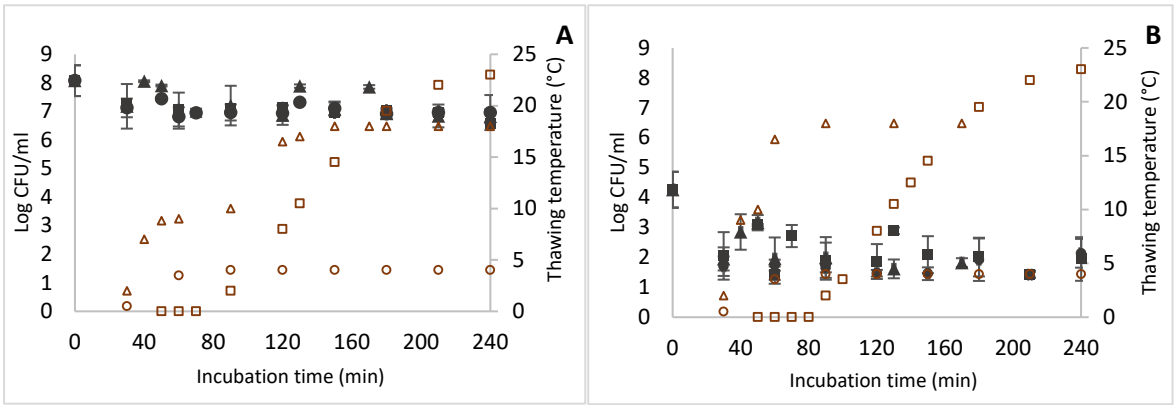

Figure 3-8. Bacterial counts during thawing at 4°C (●), 18°C (▲) and 25°C (■). Group A has high initial counts, group B has low initial counts. Open symbols represent temperature during thawing.

Histamine production by bacteria during thawing is shown in Figure 3-9. The initial concentration of histamine in Group A and B before freezing was 166.4 µg/ml and 1.2 µg/ml, respectively. The maximum concentration of histamine produced by Group A after thawing was 520.1 µg/ml, while in group B histamine produced after thawing was less than 5 µg/ml. At low thawing temperature (4°C), the histamine concentration in group A increased from 166.4 to 345 µg/ml after 1h of incubation, then decreased to 204 µg/ml after 4h of incubation. At higher thawing temperature (18 and 25°C), the histamine in group A increased gradually during incubation. Heating did not significantly reduce the concentration of histamine, although a slight decrease (186.1 µg/ml) was observed at 18°C. When high level of bacterial cells and histamine was already present before freezing (Group A), the histamine formation was continued during thawing, even though the bacterial cells did not increase.

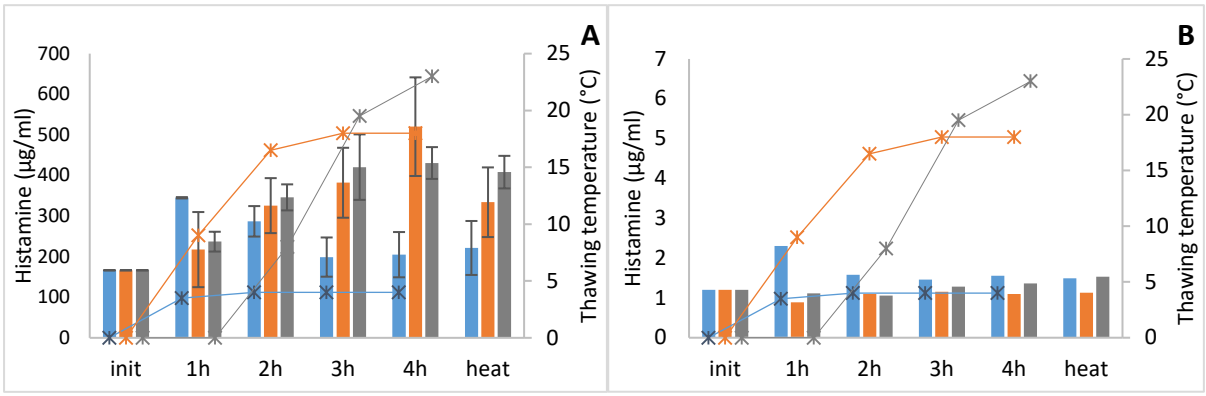

Figure 3-9. Histamine concentration during thawing at 4°C (blue bars), 18°C (orange bars) and 25°C (grey bars). Group A has high initial counts, group B has low initial counts. Lines represent temperature during thawing.

3.4. Discussion

3.4.1. Growth and histamine formation by *E. aerogenes*

Predictive models have been widely used to support microbial food safety assessment of foods by understanding the behaviour of bacteria at given conditions, evaluating the effectiveness of microbial inactivation techniques and defining the growth boundaries of specific pathogens and

spoilage microorganisms. Software or databases such as ComBase (https://www.combase.cc/index.php/en/). Pathogen Modelling Program (PMP) (https://pmp.errc.ars.usda.gov/default.aspx) and Food Spoilage and Safety Predictor (FSSP) (http://fssp.food.dtu.dk/) are available online to facilitate the prediction.

Histamine and other biogenic amines are the main sources of HFP and become important aspects of seafood product safety. Although the symptoms of HFP are generally mild and the disease is self-limited, the occurrence of HFP has been reported worldwide and caused significant health and economic losses (Hungerford, 2010; Kim et al., 2004). Predictive modelling of histamine formation has been developed in broth media (Emborg & Dalgaard, 2008b; Torres et al., 2002), skipjack tuna stored at low temperature (Frank & Yoshinaga), jack mackerel (Bermejo et al., 2004) and cold cooked salmon (Jorgensen et al., 2000). The prediction of histamine formation by *M. morganii* and *M. psychrotolerans* (Emborg & Dalgaard, 2008a, 2008b), in particular, has been incorporated into the FSSP software. Those authors used a yield factor to predict the rate of histamine formation related to the bacterial growth rate, as the histamine production depends on the number of bacterial cells (Rodriguez-Jerez et al., 1994).

In this study, a model to predict the growth and histamine formation of *E. aerogenes* was developed. Under the studied temperatures (10, 15, 20 and 30°C), the isolate has an optimum growth temperature of 30°C, without additional salt in the media. At this temperature, >100 µg/ml histamine was formed after 8 h of incubation and reached the highest level of 6,264 µg/ml at the end of incubation period (24 h). This temperature is within the suggested optimum temperature of *E. aerogenes* to grow and to produce histamine in fish, which are 25° and 37°C, respectively (Tsai et al., 2005a).

The relationship between maximum growth rate (μ_{max}) with salt and temperature was determined using multiple linear regression and Ratkowsky models. The models were evaluated based on the RMSE and R^2 values. RMSE value indicates the precision of a predictive model by

comparing the observed and predicted value of growth rate. A smaller RMSE shows more precise data described by the model (Ross, 1996; Sant'Ana et al., 2012), while a high RMSE indicates unacceptable prediction (Oh et al., 2012).The RMSE of the multiple linear regression (0.02305) was lower than the Ratkowsky model (0.0415), showing that the growth rate data of *E. aerogenes* are better fitted to the multiple linear model than the Ratkowsky model. The R^2 values of both models also showed that the multiple linear regression model has a better performance than the Ratkowsky model.

Histamine production of *E. aerogenes* showed a similar pattern as the bacterial growth curve, except a longer lag phase of histamine formation which was observed in the early incubation hours. Once the bacterial density reached approximately $10^7 – 10^8$ CFU/ml (late exponential phase), histamine was rapidly produced to more than 200 μg/ml levels. Several studies suggest that the highest production of histamine by *Morganella morganii* occurred in the middle of exponential phase (Kim et al., 2000; Omura et al., 1978; Yoshinaga & Frank, 1982), while another study found that *Tetragenococcus muriaticus* produced the highest level of histamine during the late stationary phase (Kimura et al., 2001; Takahashi et al., 2003). The type of bacteria and growth condition are factors that might influence these differences (Kimura et al., 2001). Based on these results, identification of HPB should be done as early as possible, to prevent further growth of the bacteria and the accumulation of histamine.

The histamine yield factor of *E. aerogenes* at 30°C with 1.5% NaCl from this study (5.6 -log (μg/CFU) was comparable to another study by Roig-Sagues et al. (1996) who found a yield factor of *E. aerogenes* grown in TSB with 1% L-histidine at 37°C as 5.5 -log (μg/CFU). Furthermore, the overall yield factors obtained from this study were also within the range of histamine yield factors produced by other *Enterobacteriaceae*, including *E. aerogenes*, *K. pneumonia* and *M. morganii* (López-Sabater et al., 1996; Roig-Sagues et al., 1996). However, a higher histamine yield factor (7.5 -log (μg/CFU)) was observed from *M. psychrotolerans* grown in amino acid-enriched LB broth at 2°C (Emborg, 2007). At histamine concentration higher than 1,000 μg/ml, the model underestimated the amount

of histamine produced by *E. aerogenes*. This condition occurred during the stationary phase of the bacterial growth.

At an optimum growth condition and with no additional salt in the media, *E. aerogenes* can grow and produce histamine to toxic levels, *i.e*. >200 µg/ml (FAO & WHO, 2012). Furthermore, with 6% NaCl in the medium, which represents the amount of salt added during *pindang* processing, the growth of *E. aerogenes* in broth was only prevented when incubation was done at lower temperature (10°C), while toxic levels of histamine (363 µg/ml) were still produced at higher temperature (30°C). *E. aerogenes* survived 10% NaCl only at 30°C incubation and reached a maximum density of 5.9 log CFU/ml after 120 h of incubation and produced less than 5 µg/ml of histamine. The Standard National Indonesia (SNI) suggested 20% NaCl to be used in *pindang* processing (SNI 2717.3:2009). The results from this study show that the growth of *E. aerogenes* was prevented by 20% NaCl, thus no histamine was observed at this condition. *E. aerogenes* grown in glucose, tryptone, yeast autolysate (GTY) broth with 3% added NaCl produced the highest concentration of histamine while higher salt concentration inhibited the bacterial growth causing a decrease in the amount of histidine decarboxylase enzyme in the culture media (Greif et al., 2006). The significant effect of salt on extending the lag time of *E. aerogenes* at different temperature was also observed in the current study.

Emborg and Dalgaard (2008a) used bias and accuracy factors (Ross, 1996) as indices to evaluate the performance of a mathematical model to predict the critical concentrations of histamine. The approach suggested that a model that can predict time which are 42% shorter to 25% longer than the observed time is considered as acceptable. In this study, a model to predict the time required for *E. aerogenes* to produce 100 µg/ml of histamine was obtained. The concentration chosen was based on the maximum allowable level of histamine in raw materials and cooked *pindang* of 100 mg/kg (SNI 2717.1:2009). Using the model in Equation 3-11, the predicted time to produce 100 µg/ml histamine was between 21% shorter to 24% longer than the observed time, except for incubation at 20°C, with 6% NaCl where the predicted time was 46% longer than the

observed time. The model has a calculated accuracy factor (Ross, 1996) of 1.23, *i.e.,* that predicted values are, on average, within 23% of the observed values, supporting the above observations. The bias factor (Ross, 1996) was not determined because the model is not being compared to independent data. Furthermore, the linear regression between the observed and predicted times have high R^2 value (0.9111). Therefore, the model is acceptable to use to estimate the formation of 100 µg/ml of histamine in conditions that mimic *pindang* processing. The model estimated 13.2% as the maximum NaCl concentration and 6.1°C as the minimum temperature to prevent the formation of 100 µg/ml of histamine by *E. aerogenes*. The minimum temperature was higher than the minimum temperature that limit the growth of *E. aerogenes* (3.9°C) (Equation 3-13), which showed that at lower temperature the isolate was unable to produce histamine up to 100 µg/ml. The maximum NaCl concentration estimated for histamine was also higher than the concentration that limits the bacterial growth (9.89%). The predicted times model was derived from both observed and predicted values to reach 100 µg/ml of histamine, while the growth model was obtained from observed values. This could lead to an over-estimation of a parameter, in this case NaCl concentration.

As shown from this study, the use of a higher salt concentration (10%) is effective to control the growth and histamine formation by *E. aerogenes*. However, when a lower concentration (6%) of salt is used, bacterial growth and histamine formation was only prevented at low temperature.

3.4.2. The effect of thawing on the growth and histamine formation by *E. aerogenes*

In the thawing experiment, the initial counts of *E. aerogenes* determined the level of histamine produced by the bacteria. Group A with high initial loads showed higher initial histamine concentration (>100 µg/ml) before freezing. Although a slight decrease in the viable counts and lag phase were observed from this group during thawing at 18 and 25°C for 4 h, the histamine concentration showed an increasing trend. A high concentration of histamine can be formed despite the absence of cultivable HPB, especially when freezing took place just before the levels of HPB

reached 10^7 CFU/ml (Fujii et al., 1994; Takahashi et al., 2003). Histidine decarboxyase that has been formed prior to freezing may also still be active and increases the risk of histamine accumulation during long-term freezing (ICMSF, 2005) and after thawing. Similar observations were reported by Guillén-Velasco et al. (2004) and Behling and Taylor (1982). These studies suggested a constant production of histamine when the bacterial population decreased might occur due to cell mechanism to obtain energy or due to the presence of bacterial subpopulation within the sample, which has different ability to produce histamine during the stationary or decay phase. Therefore, although thawing in still or moving air at ≤18°C is recommended (Jason, 1974), when high levels of histamine are already present, further histamine formation still possibly occurs.

At lower thawing temperature (4°C), the bacterial counts also decreased to the same levels as higher incubation temperature and showed a lag phase. However, the histamine level decreased during incubation at this temperature. Another study of *Photobacterium histaminum* showed similar results (Fujii et al., 1994). After seven days of incubation at 4°C, no viable cells of *P. histaminum* were detected, while 27% of decarboxylation activity loss was observed. It was suggested that freezing could cause damage to the bacterial cells and delay the histamine formation (Behling & Taylor, 1982; Fujii et al., 1994; Taylor, 1986). Furthermore, the group with low initial counts of *E. aerogenes* did not show significant growth and histamine formation during thawing at different temperature for 4h. The histamine formed in this group was almost negligible (<3 µg/ml)

E. aerogenes used in this study was isolated from *pindang* from Sukabumi, West Java Province, Indonesia. This bacterium is also found as a post-processing contaminant in *pindang* from tuna *(Auxis rochei)* (Fatuni et al., 2014). Furthermore, *Enterobacter* spp. isolated from *peda,* a traditional fermented fish product from Indonesia, showed the ability to produce histamine at 3,054 mg/l in broth media (Indriati et al., 2006; Mangunwardoyo et al., 2007). The role of this bacteria to cause HFP after consuming seafood has been highlighted in many studies (Björnsdóttir-Butler et al., 2010; Emborg & Dalgaard, 2006; Kanki et al., 2004; Lehane & Olley, 2000; Visciano et al., 2012).

Therefore, understanding the behaviour of *E. aerogenes* in a condition that mimics the processing of *pindang* is important to ensure adequate control of histamine formation in the product.

3.5. Conclusion

Early detection of HPB is necessary to prevent further growth and histamine accumulation. It is suggested that a combination of salt addition and low temperature gives a more pronounced effect to suppress the growth and prevent the histamine formation of *E. aerogenes* in broth media. Although freezing can reduce the viable counts of *E. aerogenes* and delay the bacterial growth at subsequent thawing, however, the pre-formed histamine cannot be eliminated by freezing and potentially increases when the product is exposed to high temperature. Therefore, preventing the growth of *E. aerogenes* should be done as early as possible, and low temperature should be maintained to avoid further formation and accumulation of histamine.

Chapter 4. Evaluating the Growth and Histamine Formation of *E. aerogenes* in Grey Mackerel (*Scomberomorus semifasciatus*) and Blue Mackerel (*Scomber australasicus*) Prepared as *Pindang*

4.1. Introduction

A previous study (Chapter 3) evaluated the growth and histamine formation of *E. aerogenes* isolated from *pindang* in broth media. Results from that chapter indicated that the isolate could grow at each tested temperatures (10, 15, 20 and 30°C), when no salt was added to the media. Therefore, a combination of low incubation temperature and 6% NaCl concentration in the media was suggested to prevent the growth and histamine formation of *E. aerogenes*.

The current study aimed to further evaluate the behaviour and histamine production of *E. aerogenes* in two Scombroid species, Grey (*Scomberomorus semifasciatus*) and Blue mackerel (*Scomber australasicus*), based on its availability in local fish markets in Tasmania. Mackerels were chosen in this study to represent the type of Scombroid fish that can also be found in tropical areas such as Indonesia. Fresh mackerel (*Scomber scombrus*) have high levels of histidine in their muscle tissue (5.5g/kg of fish flesh), thus are suitable to be used in this experiment (Fernandez-Salguero & Mackie, 1979).

An experiment to validate the growth and histamine formation models of *E. aerogenes* (Chapter 3) was done using Grey mackerel, while Blue mackerel was used to mimic the processing of *pindang*. The *pindang* processing experiment was based on a worst-case scenario to evaluate how the processing steps affected the growth of pre-inoculated *E. aerogenes* as well as the production of histamine in fish processed for *pindang*. Suggestions and intervention strategies to prevent the growth and histamine formation in *pindang* processing will be made based on results from this study.

4.2. Materials and methods

4.2.1. Culture preparation

A stock culture of *E. aerogenes* was stored at -80°C before use. Prior to the experiment, the culture was refreshed by growing in nutrient broth (Oxoid, UK) and incubating at 30°C for 24 h. One loopful of the culture was then streaked onto tryptone soya agar (TSA) and incubated at the same condition (Oxoid, UK with 1.5% agar). Prior to the experiment, a single colony of *E. aerogenes* from TSA was picked and grown in tryptone soy broth (TSB) overnight at 30°C. Serial dilutions to achieve ~10^3 – 10^4 CFU/ml were made with phosphate buffer saline (PBS), containing 0.24 g potassium phosphate monobasic (KH_2PO_4) (Merck, USA), 1.44 g sodium phosphate dibasic ($Na_2HPO_4.7H_2O$) (Merck, USA), 8 g sodium chloride (NaCl) (Merck, USA) per litre of distilled water, with pH adjusted to 7.4.

4.2.2. Artificial contamination of fish

Grey mackerel (*Scomberomorus semifasciatus*) was cut into small pieces of approximately 5 g and weighed. Fish pieces were then treated with 6% NaCl (w/w) by evenly distributed salt above the fish surface, to represent the amount of salt added in the processing of *pindang*. No salt treatment was used as a control treatment. After that, each individual cut was dipped into the bacterial culture for 1 min and left for another 2 min to dry the excess dipping solution. Fish pieces were placed in sterile Falcon tubes and incubated at different temperatures. For no salt treatment, samples were incubated at 30°, 20°, 15° and 10°C for 28 h, 42 h, 6 d, and 16 d, respectively. While fish treated with 6% of salt was incubated at 30° and 20°C for 35 h and 4 d, respectively.

For the experiment that represented the thawing method in the processing of *pindang*, Blue mackerel (*Scomber australasicus*) that weighed 300 – 400 g per individual sample was used. Fish samples were dipped into the bacterial suspension for 30 min and left for 5 min to allow the excess liquid to dry. Fish samples were then placed in sterile plastic bags and stored at -20°C for 3 d. A worst-case scenario was obtained based on results from the previous experiment (Chapter 3). Fish

thawing was done at 25°C for 4 h and samples were taken at 2 and 4 h. After that, fish samples were washed with clean water and arranged in a cooking container. Water used in the processing was obtained from a protected dug well that is classified as an "improved drinking-water sources" (WHO & UNICEF, 2010). The improved sources are defined as drinking water sources that are protected from contamination, especially faecal contamination. These sources are including piped households, public taps, tube wells or boreholes, protected dug wells, and protected springs or rainwater (WHO & UNICEF, 2010). Fish samples were then supplemented with 6% NaCl (w/w), then steamed (boiled) for 4 h. The temperature during cooking was recorded. The cooked fish was packed in sterile bags and stored at 4 and 25°C for 5 d.

Water activity, microbiological and histamine samples were taken regularly as described in section 4.2.3 and 4.2.4.

4.2.3. Microbiological analysis

Five gram of fish flesh was homogenized in 45 ml of PBS. The homogenate was then serially diluted in PBS and 100 µl was spread-plated onto Niven agar (Niven et al., 1981), containing 0.5% tryptone (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 0.5% sodium chloride (Merck, USA), 2.7% L-histidine (Sigma, USA), 2% agar and 0.006% bromcresol purple, with pH adjusted to 5.5 – 5.7. The plates were incubated at 30°C for 24 – 36 h.

For the total viable count (TVC), serial dilutions of culture in PBS were spread-plated onto tryptone soya broth (Oxoid, UK) with added 1.5% agar and 2% NaCl, and incubated at 30°C for 24 h. Colonies of total viable aerobic cells were counted and expressed as log CFU/g.

4.2.4. Histamine analysis

Sample preparation and chromatography analysis of histamine followed the procedure described in Chapter 3 of this thesis.

4.2.4.1. Histamine extraction from fish sample

The procedure for histamine determination followed the methods of Sirocchi et al. (2014), with modification. Fish flesh was blended and 5 mg was extracted with 15 ml of 15% trichloroacetic acid (TCA) (Sigma, USA). The homogenate was centrifuged at 2,500 x *g* for 10 min and approximately 10 ml of the supernatant was collected in a new tube. After that, 100 µl of 10 µg/ml of histamine.2HCl (α, α, β, β-D4, 98%) (Novachem, AU) was added into the supernatant. Sample pH was adjusted by adding 200 µl of 28% NH_4OH (Sigma, USA).

Sample clean-up was performed using SPE STRATA X cartridge 33 µm polymeric reverse-phase, 30 mg/3 ml (Phenomenex, AU). Prior to sample loading, the cartridge was conditioned with 2x2 ml of methanol followed by 2x2 ml of Milli-Q water. Two ml of sample was loaded into the cartridge then rinsed with two ml of a mixture of MeOH/H_2O (5/95 *v/v*). The cartridge was dried under vacuum to remove the excess water. The analyte was eluted twice with 2 ml methanol/acetic acid (99/1 *v/v*) and dried with nitrogen gas. Analyte was re-dissolved in two ml of Milli-Q water. Prior to the chromatography analysis, the samples were acidified with 0.1% formic acid (1:1 v/v).

4.2.4.2. Chromatography analysis

A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was coupled with a Waters Acquity UPLC PFP column (2.1 mm × 100 mm × 1.7 µm) for analysis. The mobile phase consisted of two solvents: 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). The UPLC program was 100% A to 60% A:40% B at 3.0 min, which was held for 0.5 min, and this was followed by immediate re-equilibration to starting conditions for 3 min. The flow rate was 0.20 ml/min. The columns were held at ambient temperature, and the sample compartment at 6°C. Injection volume was 10 uL. Approximate retention time for histamine was 2.9 min.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.8 kV, and multiple reactions monitoring (MRM) was used to detect all analytes. For

histamine, the MRM transitions of the precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 95.0 $[M+H]^+$ was used for quantitation with a dwell time of 22 msec, cone voltage was 21 V and collision energy 12 V; precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 68.0 $[M+H]^+$ was used for confirmation, cone voltage 21 V and collision energy 20 V. For D4-histamine the MRM transitions of the precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 99.0 $[M+H]^+$ was used for quantitation; precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 72.0 $[M+H]^+$ was used for confirmation, with the same cone voltages and collision energies. The ion source temperature was 130°C, the desolvation gas was N_2 at 950 l/h, the cone gas flow was 100 l/h, and the desolvation temperature was 450°C. Data were processed using MassLynx software (Waters Corp, 2018).

4.2.5. Modelling the growth and histamine formation of *E. aerogenes* in fish

Growth curves for each temperature and salt combination were obtained using the primary data-fitting model of Baranyi and Roberts (Baranyi & Roberts, 1994) in DMFit Software Version 3.5 (www.combase.cc).

$$ln\big(N(t)\big) = ln(N_0) + \mu_{max}A(t) - ln[1 + \frac{e^{\mu_{max}A(t)}-1}{e^{(N_{max}-N_0)}}$$ (Equation 4-1)

$$A(t) = t + \frac{1}{\mu_{max}}\ln(\frac{e^{(-\mu_{max}t)}+q_0}{1+q_0})$$ (Equation 4-2)

$$\lambda = \frac{\ln(1+\frac{1}{q_0})}{\mu_{max}}$$ (Equation 4-3)

where:

$ln\big(N(t)\big)$ = log of cell concentration at time *t* (h), in CFU/g; $ln(N_0)$ = log of initial cell concentration, in CFU/g; $ln(N_{max})$ = log of maximum cell concentration; μ_{max} = maximum growth rate (h^{-1}); and λ = lag time (h).

The kinetic data obtained from this study were deposited in ComBase (www.combase.cc) following publication of the thesis under the source name “Rachmawati”.

An extended Ratkowsky models (McMeekin et al., 1987) (Eq. 4-4) was used to describe the relationship between growth rate and storage temperature.

$$\sqrt{\mu} = b\ (T - T_{min})\sqrt{(a_w - a_{wmin})} \quad \text{(Equation 4-4)}$$

where:

μ_{max} = maximum growth rate, b = constant value, T = temperature (°C), T_{min} = theoretical minimum temperature that supports the growth (°C), a_w = water activity (%), a_w = theoretical minimum a_w that supports the growth (%).

The rate of histamine formation was estimated using a yield factor (Emborg & Dalgaard, 2008a, 2008b; James et al., 2013; Jorgensen et al., 2000).

$$His(t) = His(t_0) + Y_{His/CFU}\, x\big(CFU(t) - CFU(t_0)\big)x1000 \quad \text{(Equation 4-5)}$$

$$pY_{His/CFU} = -log\left(\frac{His(final) - His(t_0)}{CFU(final) - CFU(t_0)}\right) \quad \text{(Equation 4-6)}$$

where:

$pY_{His/CFU}$ is the constant yield factor; $CFU\ (t_0)$, $CFU(final)$ and $CFU(t)$, are colony forming units per ml at initial, final and time (t), respectively; $His(t_0)$, $His(final)$ and $His\ (t)$ are histamine concentration in µg/g at initial, final and time (t), respectively.

Significant differences (P<0.05) between the kinetic parameters were analysed using analysis of variance (ANOVA) in SigmaPlot Version 12.5.

4.3. Results

4.3.1. Model validation of *E. aerogenes* growth and histamine formation in fish

The water activity values of fish during storage were between 0.95 – 1 (Figure 4-1). At 6% NaCl, the water activity of fish was lower than fish without salt. Lower water activity also observed at lower incubation temperatures.

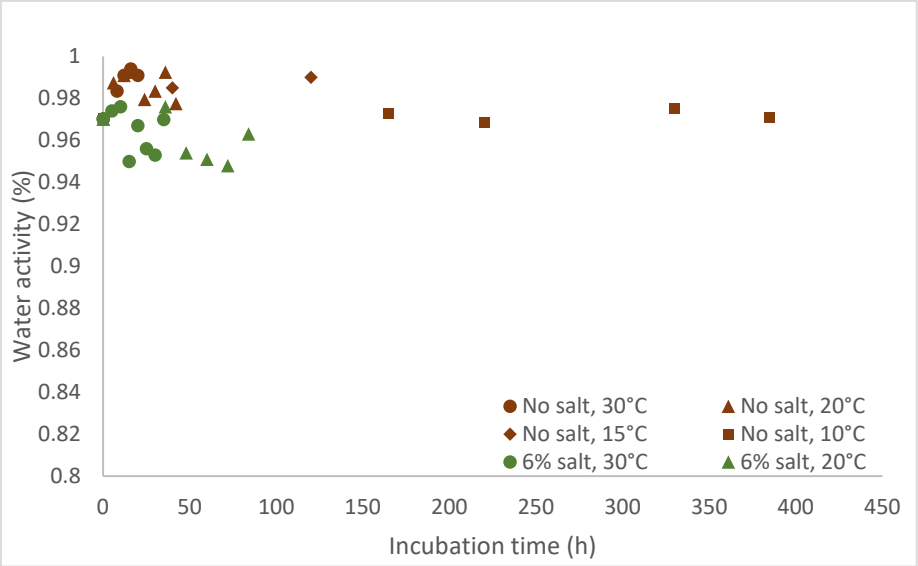

Figure 4-1. Water activity of fish during incubation

The *E. aerogenes* isolate grew at all treatment combinations (Figure 4-2), with an average initial and final count of 2.48 ± 1.4 and 8.68 ± 0.55 log CFU/g, respectively. The highest density of 9.34 log CFU/g was reached in fish incubated at 30°C for 28 h, without salt addition.

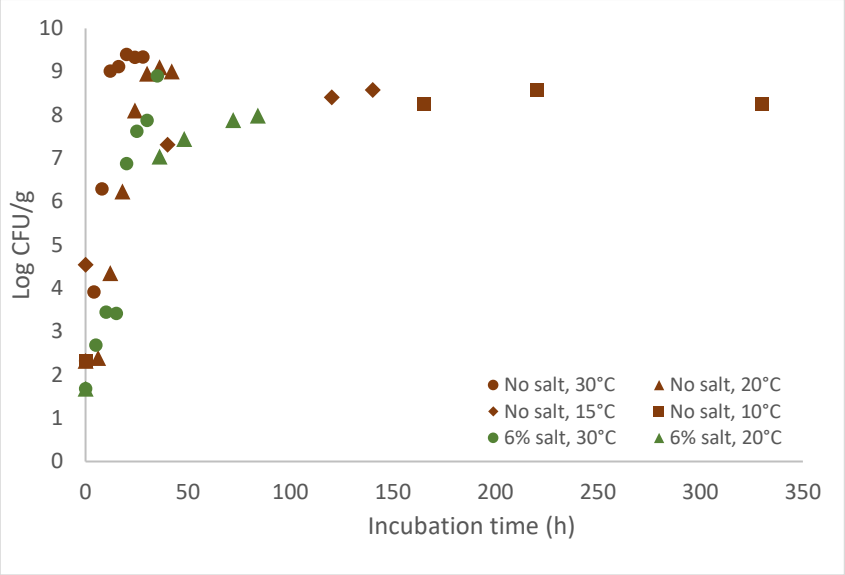

Figure 4-2. Growth curve of *E. aerogenes* during storage at different temperature and salt concentration

A similar trend was observed from the TVC during the storage experiment (Figure 4-3). The average initial count of total microflora in fish was 3.98 ± 1.1 log CFU/g, higher than initial *E. aerogenes* count. The average maximum population was also higher than *E. aerogenes*, at 9.02 ± 0.31 log CFU/g. The highest density of TVC was 9.5 log CFU/g, which was obtained after incubation at 30°C for 20 h, without NaCl.

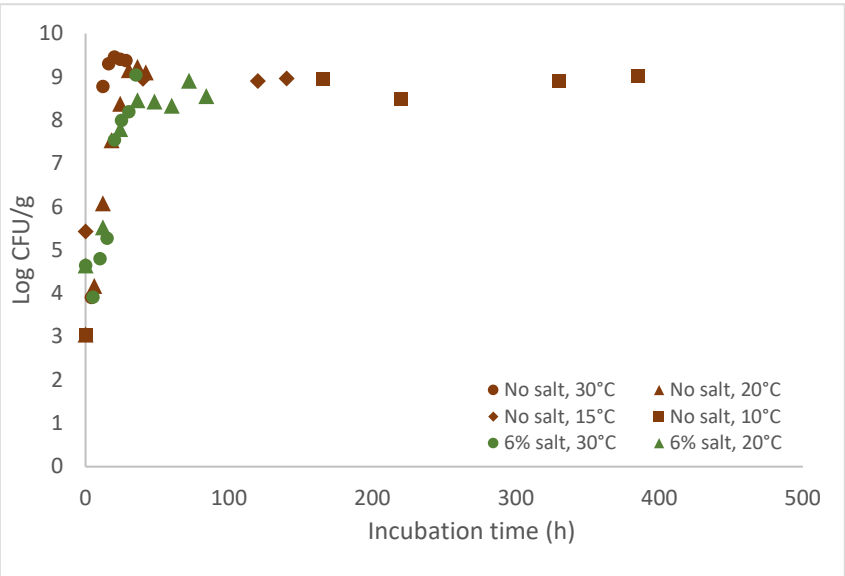

Figure 4-3. Total viable counts during fish incubation

Table 4-1 shows the growth parameters of *E. aerogenes* during incubation in fish. The maximum specific growth rate of *E. aerogenes* in fish was significantly affected by incubation temperature and salt concentration except that with no added NaCl; there was no significant difference of growth rates between 10 and 15°C.

Table 4-1. Growth parameter of *E. aerogenes* during incubation, as observed from Niven agar

Salt concentration	Temperature (°C)	λ (h)	μ_{max} (h^{-1})	N_{max} (CFU/g)
No salt	10	-	0.0121 ± 0.001 [a]	-
	15	-	0.0296 ± 0.003[a]	-
	20	5.8 ± 2	0.345 ± 0.053[b]	9.06 ± 0.2[a]
	30	2.9	0.677 ± 0.161[c]	9.33 ± 0.01[a]
6%	20	-	0.0395 ± 0.012[ad]	-
	30	-	0.207 ± 0.017[bd]	-

Different letters denote significant differences (P < 0.05)

Using the extended Ratkowsky model, the relationship between the maximum growth rates and temperatures is shown in equation 4-7. The RMSE and R^2 values of the model were 0.0923 and 0.9034, respectively. Using the equations, observed and predicted values of *E. aerogenes* growth rate in broth (Chapter 3) compare to fish are presented in Figure 4-4.

$$\sqrt{\mu_{max}} = 0.1821\ (T - 5.8917)\sqrt{(a_w - 0.9539)} \qquad \text{(Equation 4-7)}$$

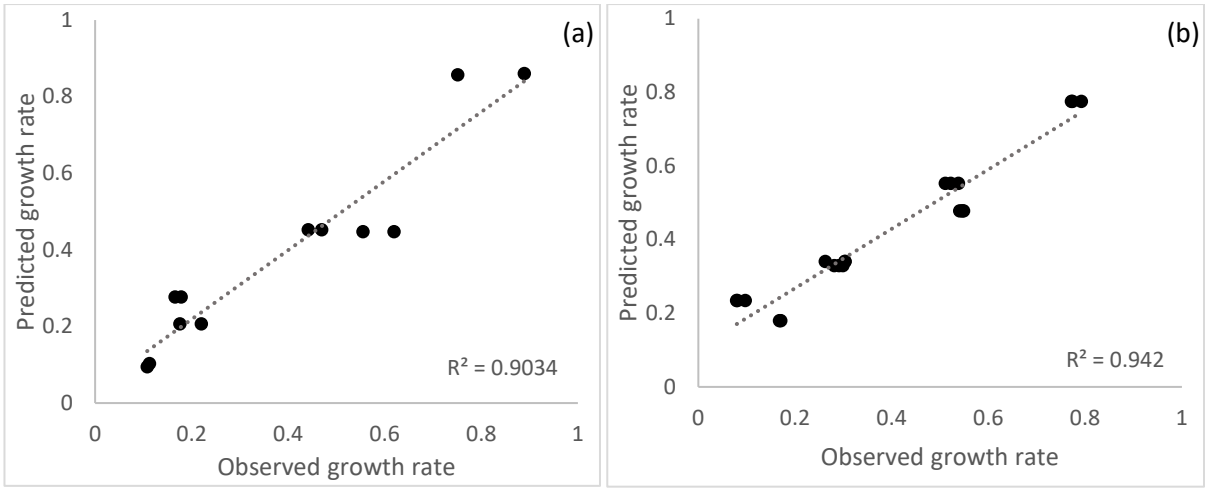

Figure 4-4. The squared-root of observed and predicted growth rate (μ_{max}) of *E. aerogenes* in (a) fish and (b) broth during storage

The amount of histamine produced during fish incubation is shown in Figure 4-5. The highest concentration of histamine was produced during incubation without salt addition, while the addition of 6% NaCl significantly lowered the amount of histamine produced by the isolate. At a lower incubation temperature (10°C), the histamine formation was delayed and 385.8 µg/g of histamine was only detected after approximately two weeks.

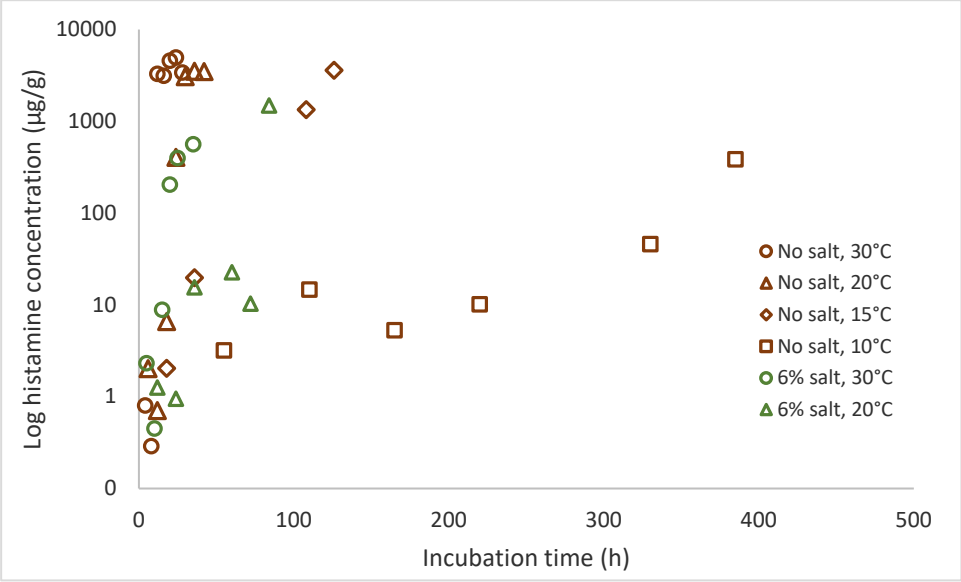

Figure 4-5. Histamine concentration during fish incubation at different temperature

The histamine yield factors obtained from each treatment are presented in Table 4-2. Although the yield factors were not significantly different between treatment, the yield per cell for bacteria incubated at 10°C was higher than the other incubation temperatures (with 1.5% added NaCl). At 6% NaCl, the histamine yield factor at 20°C was higher than at 30°C. The observed versus predicted histamine is presented in Figure 4-6.

Table 4-2. Yield factor ($pY_{His/CFU}$) of histamine in fish during incubation

Salt (%)	Temperature (°C)	*pYhis/CFU* (-log(µg/CFU))
1.5	10	6.549 ± 1.5
	15	5.462 ± 0.82
	20	5.527 ± 0.06
	30	5.663 ± 0.12
6	20	5.041
	30	4.643 ± 1.5

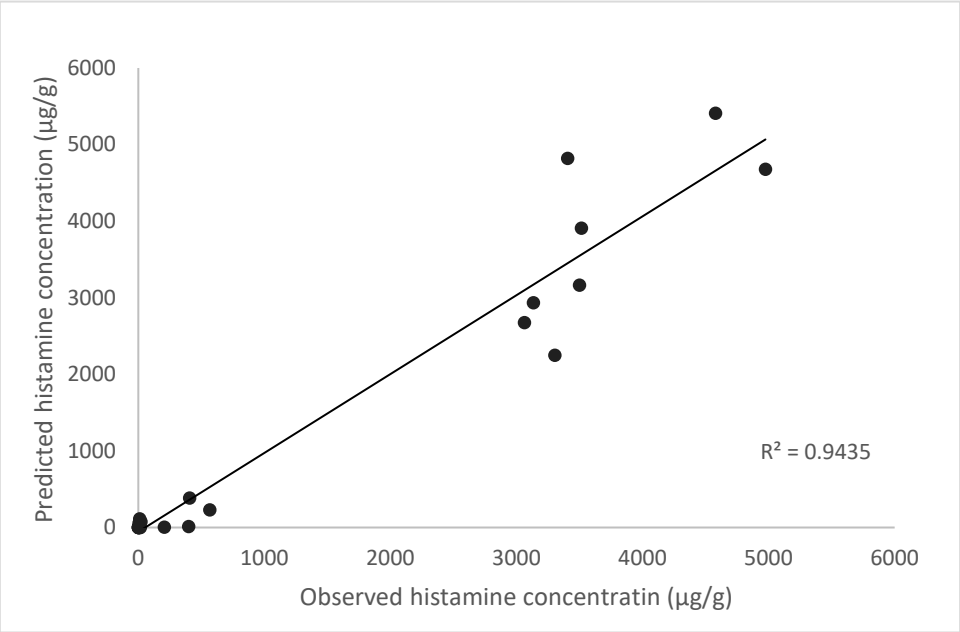

Figure 4-6. Predicted and observed histamine concentration produced by *E. aerogenes* in fish

4.3.2. Investigation of *E. aerogenes* growth and histamine formation in mackerel under *pindang* processing condition

The water activity of raw mackerel varies during fish preparation and after cooking. Raw mackerel used in the experiment have aw of 0.993. However, during preparation steps (thawing, washing and salting) the water activity decreased to 0986. The levels decreased after cooking (0.979) and increased again during fish storage at 4°C and 25°C.

The total count of *E. aerogenes* and histamine concentration during *pindang* preparation is shown in Figure 4-7. No typical HPBs colonies were observed in Niven agar from the fresh fish samples. Following the artificial contamination, the number of HPB colonies in Niven agar was 4.5 log CFU/g of fish. This value was lower than the density of *E. aerogenes* in the dipping solution, which was 7.7 ± 0.2 log CFU/ml. After fish was frozen, the total number of presumptive *E. aerogenes* decreased, although not significantly, during the preparation steps (dipping and thawing). However, no growth was observed after cooking for approximately 3 h. When fish was stored at 25°C, bacterial growth was re-activated after 2 d and reached 5 log CFU/g. Moreover, at lower temperature (4°C), the bacterial growth was observed only after 5 d of storage.

The histamine formation showed similar trends as the total count of *E. aerogenes*, except that the levels of histamine remained high after the cooking step. Storage temperatures did not affect the accumulation of histamine, as the histamine levels at the end of the storage trial were 752.7 and 568.7 µg/g, for fish stored at 25 and 4°C, respectively.

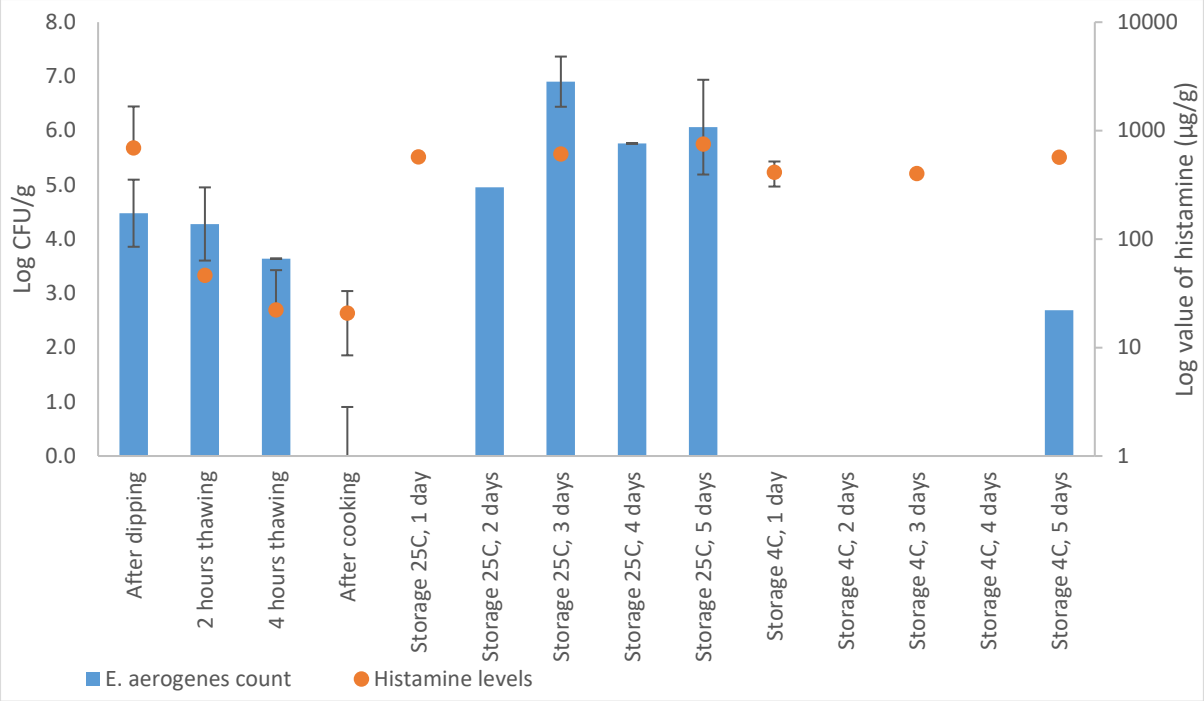

Figure 4-7. *E. aerogenes* and histamine levels during the preparation of salted-boiled mackerel (bar symbols represent the *E. aerogenes* cell counts (log CFU/g), dot symbols represent the histamine concentration (µg/g).

4.4. Discussion

Histamine formation in fish muscle occurs post-mortem (Hongpattarakere et al., 2016; Lehane & Olley, 2000; Shakila et al., 1996). When histamine is formed before proteolysis of fish muscle in the post-mortem period, it is difficult to identify high levels of histamine in fish muscle based on the organoleptic properties of the fish, and this increases the possibility of consuming contaminated fish (Lehane & Olley, 2000; Visciano et al., 2012). Therefore, early detection of histamine, or prevention of its formation, is necessary to ensure the safety of the fish products for consumption.

Model validation in fish showed that the extended Ratkowsky model performed better in predicting the growth of *E. aerogenes* in broth experiment than in fish. As shown in Figure 4-4, the linear correlation between μ_{max} observed and μ_{max} predicted in broth was higher (R^2 = 0.942) compared to fish (R^2 = 0.9034). At similar incubation conditions, μ_{max} from the broth experiment was

generally higher than in fish experiment, except at higher temperature (20 and 30°C) without NaCl, the isolate grew faster in fish. The slow growth of bacteria in fish might be due to competition between the target isolate with the background microflora and to bacterial stress caused by the fish matrix (Sant'Ana et al., 2012). Jeanson et al. (2015) suggested that the bacteria in solid food are present in a colony-immobilized form and their growth depends on the diffusion of substrate through the matrix. In contrast, the substrates are evenly distributed in a culture media, and thus the bacteria can grow faster.

For the validation experiment, no HPB were identified and a negligible amount (< 5 µg/g) of histamine was detected in fresh Grey tuna, showing that the fish used in the experiment was not contaminated with histamine from other sources. Several studies also reported that histamine is not found in fresh tuna muscle (López-Sabater et al., 1996; Silva et al., 1998). During the incubation, the growth of *E. aerogenes* and the TVC showed similar trends, which is possibly due to similar ability of other natural fish microflora to grow under the same conditions. Several studies show that the addition of salt to fish products may trigger the growth of the fish's natural microflora, especially lactic acid bacteria and *Enterobacteriaceae* (Gram et al., 2002; Hansen & Huss, 1998). However, at lower incubation temperature and with the presence of 6% NaCl, the maximum population of *E. aerogenes* in fish was lower than the TVC. This result was in accordance with result from broth experiment, where *E. aerogenes* reached only 7.7 and 7.5 log CFU/ml at the end of incubation, when 6% of NaCl was added into the media (Chapter 3, Result section). This condition is known as Jameson effect, *i.e.* the maximum density of a particular microorganism is supressed by an overgrowing microflora (Gram et al., 2002; Jameson, 1962). The slow growth of *E. aerogenes* in higher salt condition might correspond the multiplication of other halophilic bacteria from the fish which represented with higher TVC. Wendakoon and Sakaguchi (1995) showed that 2 and 3% NaCl could reduce the growth rate of *E. aerogenes* in mackerel

At the same incubation temperatures and with 6% NaCl, the histamine formed in fish was much higher than in the broth (Chapter 3). For example, at 30°C with 6% NaCl, *E. aerogenes* only

produced 363 µg/ml of histamine in broth media after 33 h, while approximately 400 µg/g of histamine was already found in mackerel after 25 h. This might be due to high amount of free histidine present in the fish muscle. Sterile mackerel muscle contains 5.5 g/kg histidine (Fernandez-Salguero & Mackie, 1979), while in another Scombroid fish (tuna), the histidine level could be as much as 15g/kg (Lehane & Olley, 2000). The broth however only contained 5 g of l-histidine per litre.

However, similar trends of histamine formation were observed from both experiments. At lower temperature and higher salt concentration, the concentration of histamine produced was lower. As observed in other studies, higher salt concentration may reduce the concentration of histamine in fish. The addition of 10% salt in miso fermentation process reduced the histamine levels from more than 100 µg/g to less than 50 µg/g more when only 5% salt was added (Chin & Koehler, 1986). Moreover, a combination of high salt concentration and low storage temperature was suggested to be used to prevent the formation of histamine in salted anchovies (Karaçam et al., 2002; Veciana Nogués et al., 1997).

The yield of histamine produced per cell of bacteria showed no significant differences between different incubation conditions, while at lower incubation temperature the histamine production was slightly more rapid than at higher temperature. A similar observation was found by Bermejo et al. (2004), where the histamine yield of jack mackerel (*Trachurus symmetricus*) incubated at 5°C was larger those incubated at 15 and 25°C. This condition might be due to the reduced activity of amine oxidase at lower temperature, thus slower the degradation rate of histamine in the fish muscle (Bermejo et al., 2004). Furthermore, the yield values observed in this study ranged between 4.643 ± 1.5 to 6.549 ± 1.5 -log (µg/CFU), which is similar to the yield value of histamine (5.0 ± 0.2 -log (µg/CFU)) produced by *P. phosphoreum* in cold-smoked salmon stored at 5°C (Jorgensen et al., 2000). The histamine model was better predicted the amount of histamine produce by *E. aerogens* in fish, compared to broth experiment (Chapter 3). However, not all histamine curves can be fitted to the model, especially from incubation 20°C with 6% NaCl.

Field observation showed that *pindang* made from frozen fish have higher risk of being contaminated with HPB and histamine (Chapter 2 and 5). Therefore, a worst-case scenario of *pindang* processing using frozen raw material was investigated to evaluate how the current processing of *pindang* influences the histamine levels of the final product. High initial counts of *E. aerogenes* and high histamine levels of raw mackerel were chosen as initial parameters in this scenario. Fresh Blue mackerel was artificially contaminated with *E. aerogenes* to promote the histamine formation in fish flesh. The contamination that took place before freezing was done to simulate pre-contamination of fish which may occur before fish was transported to processing facilities. Thawing at 25°C mimics the current thawing condition during *pindang* processing, where refrigeration or cooling systems were not available. Based on the current processing practice of *pindang*, the amount of salt added was 6% (w/w). This concentration is lower than the recommended amount of salt suggested by SNI (10 – 20%). However, results from Chapter 3 showed that 6% NaCl addition if combined with low temperature treatment (<15°C) will be adequate to prevent the growth and histamine formation of *E. aerogenes* in broth.

Freezing has been widely used in food preservation, since this method allows the formation of ice crystals from the constituent water in cells, thus reduces the availability of water and prevents microbial growth (Attrey, 2017; Ersoy et al., 2008). Furthermore, different chemical reactions are also slowed or stopped at temperatures below zero (Attrey, 2017). Prior to serving, frozen foods may need to be thawed, or alternatively the food should be cooked two times longer than normal cooking time, if thawing is not necessary (Hicks, 2016).

In a thawing process, heat transfer occurs from water or air to melt the ice that was formed in fish muscle during freezing. The ice crystals are completely converted into water when the temperature of whole fish reaches -1°C (Archer et al., 2008). Thawing becomes critical from a food safety point of view because freezing may not completely kill the bacteria that are present in the food. Therefore, during thawing, when frozen food is exposed to warm temperatures (above 4°C), the bacteria can start to re-grow and multiply (Attrey, 2017). The slimy surface of thawed fish also

provides a good environment for bacteria to grow (Archer et al., 2008). In seafood processing, temperature abuse most likely occurs during freezing and thawing, and this condition has been known as the main causes of histamine formation. Ndraha et al. (2018) defines temperature abuse as condition where temperatures during food handling varies into unacceptable levels from the optimal temperature suggested for those food products and the variations occur for certain period of time.

In this study, fish was exposed to temperature abuse by thawing the frozen fish at 25°C for four hours. During this time, a slight reduction of *E. aerogenes* viable count was observed. However, the decrease was insignificant and possibly due to the bacteria leaching into the melted water during the thawing process. The artificial contamination was done by dipping the mackerel into a culture of *E. aerogenes*, therefore some of the bacterial cells that attached to the fish skin might be released when the outer layer of the frozen fish was exposed to heat and melted (Ersoy et al., 2008). Other studies also showed that HPB in fish was mostly present in the gill and skin (Hongpattarakere et al., 2016; Kim et al., 2003a). As the optimum growth temperature of *E. aerogenes* is 25 – 37°C (Tsai et al., 2005a), the bacterial count reaches almost the same level as the initial count (before freezing) after fish was thawed for four hours. This observation supports previous studies suggesting that bacteria may revive from a frozen condition.

From this experiment, it is clear that rapid thawing and maintaining low temperature during fish handling and preparation is essential to avoid bacterial multiplication and histamine formation. Ersoy et al. (2008) suggested to maintain the ambient temperature during thawing below 15°C to minimise bacterial growth. This condition in particular, is important to be applied in conventional thawing using circulated water or air, as the room temperature will also affect the thawing rate. Furthermore, CODEX suggest to thaw fish blocks with a maximum air temperature of 25°C or 21°C if thawing with water immersion (FAO & WHO, 1989). Another recommendation for frozen tuna handling was given by the US FDA. For tuna that has been frozen for more than six months then

exposed to temperature above 20°C, the fish should be processed for not more than 12 h (US FDA, 2011).

Decreasing levels of histamine was observed during thawing. Due to the water-soluble property of histamine, this compound might be dissolved in the melting water and released from the fish (Hongpattarakere et al., 2016; Shakila et al., 2005). Despite the effectiveness of cooking in reducing or eliminating the number of viable bacteria, the amount of pre-formed histamine remained similar during processing. This result supports previous studies which showed that histamine cannot be eliminated by common cooking practices such as freezing and heating (Kim et al., 2002; US FDA, 2011).

The cooking temperature reached more than 90°C after 90 min and remained at that level until cooking was completed (4h). The cooking step was effectively reduced the HPB, as no colonies were observed. However, storing the cooked fish at 25°C provides favourable conditions for any HPB that survive heating or HPB that are present as post-processing contaminants to continue growing, while storage at 4°C prolongs the lag period of the bacteria up to 5 d. Similar observation was reported by Lopez-Sabater et al. (1994) who studied the effect of handling on the presence of HPB and histamine accumulation in tuna destined for canning. Their study showed that pre-cooking at 100-105°C for 110 min significantly reduced the HPB counts, but the counts were increased when tuna was held at room temperatures, which due to the recovery of thermal-injured bacteria or the presence of environmental recontamination. Naila et al. (2010) also suggested that recontamination and temperature abuse could lead to the histamine formation in thermally processed products.

Furthermore, increasing concentration of histamine in fish during storage in both temperatures shows that cold storage did not effectively prevent the histamine formation, when high concentration of HPB were already present in fish.

4.5. Conclusion

The growth and histamine formation of *E. aerogenes* in fish is faster than in broth and is affected by temperature and salt concentration.

To improve the quality and safety of *pindang,* the used of good quality raw materials that meet the requirement from the Indonesian food safety authorities is recommended. The use of fish that has been pre-contaminated with HPB and histamine should be avoided. When fresh raw material is not available, frozen fish might be used as an alternative. However, thawing of the frozen fish should occur at less than 15-18°C, and the subsequent processing step should be done without delay. Furthermore, the combination of NaCl (at least 6%) and low temperature (<15°C) is effective to be used in *pindang* processing, to prevent the growth of HPB and avoid histamine formation.

Chapter 5. Field Observation on the Processing of Indonesian *Pindang* and Evaluation of Histamine Formation and Microbial Communities during the Processing.

5.1. Introduction

Pindang is an Indonesian traditional fish product which is usually made from Scombroid fish, such as tuna, mackerel and scad. The process combines salting and steaming or boiling which serve as preservatives. Variations on the processing of *pindang*, including raw material, salt concentration, boiling time, and packaging materials, depend on the common practices and the taste preferences of societies in different regions in Indonesia. These variations determine the characteristics of the product, including its organoleptic appearance and shelf life (Wibowo, 1996). According to the processing technique, the Indonesian National Standard (SNI) categorises *pindang* into brined and salted *pindang* (SNI 2717.3:2009) (BSN, 2009c). Brined *pindang* is prepared by soaking the fish in 5-10% brine then boiling it in 10% brine for 30 - 60 min, while salted *pindang* is made by adding salt on the fish surface before boiling it in water for 1 - 4 h with high heat and continue with low heat for an additional one hour.

Pindang processors in Sukabumi are family or neighbourhood based industries equipped with basic and traditional processing facilities. The processing techniques have been passed between generations. The processors usually use fresh tuna from local catchments to make *pindang.* However, frozen tuna is mostly used at the moment as an alternative raw material due to the limited number of tuna caught from local waters. In 2012, 15-20% of the total raw materials used in *pindang* processing were obtained from imported fish: most of it was frozen (Anonymous, 2012a).

Several records indicated *pindang* as the major causative agent of HFP outbreaks in Indonesia (Ainun, 2015; Anonymous, 2004, 2008, 2014, 2016). HFP is a foodborne intoxication due to the consumption of fish or other foods containing high levels of histamine (FAO & WHO, 2012;

Taylor, 1986; Taylor et al., 1989). Scombroid fish such as tuna and albacore (*Thunnus*), skipjack (*Katsuwonus*), mackerel (*Scomber*) and bonitos are known to have high levels of free histidine in their muscle and have been associated with HFP cases (Taylor, 1986).

Histamine in fish can be formed as a result of bacterial growth. A previous study (Chapter 2 of this thesis) showed the presence of histamine producing bacteria (HPB) such as *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis, Hafnia paralvei*, *Klebsiella* sp., *Citrobacter* sp. and *Providencia* sp. from *pindang* from Pelabuhan Ratu. Temperature abuse and mishandling of fish during processing, storage and distribution enable the growth of these bacteria in fish products (Baylis, 2006; FAO & WHO, 2012; Hungerford, 2010; Taylor, 1986; Taylor et al., 1989). Therefore when frozen tuna is used as the raw material in *pindang* processing, maintaining low fish temperature during the thawing step is essential to prevent bacterial growth and histamine formation.

In a food production system, the Codex Alimentarius General Principles of Food Hygiene recommends the application of a Hazard Analysis Critical Control Point (HACCP) approach to provide and enhance food safety and quality (Whitehead & Orriss, 1995). The HACCP system can be used to systematically identify, measure and control specific hazards in food production and to ensure the safety of food (FAO & WHO, 1999; FAO & WHO, 2009a). In Indonesia, the application of food hygiene practices as well as HACCP has been done in the majority of food exporting companies. However this approach could also be applied in traditional fish processors, including *pindang* processors, with some adjustment to preserve the traditional values of these small-scale industries (Heruwati, 2002).

This study aimed to describe the existing processing practices of *pindang* in Pelabuhan Ratu, Sukabumi District, West Java Province, Indonesia and how it affects the levels of histamine and the composition of the microbial community in the final products. To assist the processors to improve

their processing practices, potential hazards and CCPs which affect histamine formation and accumulation during *pindang* processing were determined.

5.2. Materials and methods

5.2.1. Fish sample collection and field observation

Field observations and sample collection were done at traditional fish processors in Pelabuhan Ratu, Sukabumi District, West Java Province, Indonesia. Five *pindang* processors were selected and the processing steps were documented. Fish temperature during processing was recorded. Raw and cooked fish from different processing steps were collected for microbiological and histamine analysis.

5.2.2. Microbiological analysis

Fifty milligram of fish flesh was homogenized in 45 ml of phosphate buffer saline (PBS), containing monobasic potassium phosphate (KH_2PO_4) (Merck, USA), dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) (Merck, USA), sodium chloride (NaCl) (Merck, USA) and distilled water, with pH adjusted to 7.4. The homogenate was serially diluted in PBS and 100 µl was spread plate onto tryptone soya broth (Oxoid, UK) with added 1.5% agar and 2% NaCl, and incubated at 30°C for 24 h. Colonies of total viable aerobic cells were counted and expressed as log CFU/g.

5.2.3. Histamine analysis

5.2.3.1. Histamine extraction from fish sample

The extraction procedure of histamine followed the methods of Sirocchi et al. (2014), with modification. Fish flesh was blended and 5 mg was extracted with 15 ml of 15% trichloroacetic acid (TCA) (Sigma, USA). The homogenate was centrifuged at 2,500 x *g* for 10 min and approximately 10 ml of the supernatant was collected in a new tube. After that, 100 µl of 10 µg/ml of histamine.2HCl (α, α, β, β-D4, 98%) (Novachem, AU) was added into the supernatant. Sample pH was adjusted by adding 200 µl of 28% NH_4OH (Sigma, USA).

Sample clean-up was performed using SPE STRATA X cartridge 33 µm polymeric reverse-phase, 30 mg/3 ml (Phenomenex, AU). Prior to sample loading, the cartridge was conditioned twice with 2 ml of methanol followed by 2 ml of Milli-Q water. Two ml of sample was loaded into the cartridge then rinsed with two ml of a mixture of MeOH/H_2O (5/95 *v/v*). The cartridge was dried under vacuum to remove the excess water. The analyte was eluted twice with 2 ml methanol/acetic acid (99/1 *v/v*) each time and then dried with nitrogen gas. Analyte was re-dissolved in two ml of Milli-Q water prior to chromatography analysis.

5.2.3.2. Chromatography analysis

A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was coupled with a Waters Acquity UPLC PFP column (2.1 mm × 100 mm × 1.7 µm) for analysis. The mobile phase consisted of two solvents: 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). The UPLC program was 100% A to 60% A:40% B at 3.0 min, which was held for 0.5 min, and this was followed by immediate re-equilibration to starting conditions for 3 min. The flow rate was 0.20 ml/min. The columns were held at ambient temperature, and the sample compartment at 6°C. Injection volume was 10 µL. Approximate retention time for histamine was 2.9 min.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.8 kV, and multiple reactions monitoring (MRM) was used to detect all analytes. For histamine, the MRM transitions: precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 95.0 $[M+H]^+$ was used for quantitation with a dwell time of 22 msec, cone voltage was 21 V and collision energy 12 V; precursor (m/z) 112.1 $[M+H]^+$ to product (m/z) 68.0 $[M+H]^+$ was used for confirmation, cone voltage 21 V and collision energy 20 V. For D4-Histamine the MRM transitions: precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 99.0 $[M+H]^+$ was used for quantitation; precursor (m/z) 116.1 $[M+H]^+$ to product (m/z) 72.0 $[M+H]^+$ was used for confirmation, with the same cone voltages and collision energies. The ion source temperature was 130°C, the desolvation gas was N_2 at 950 l/h, the cone gas flow was

100 l/h, and the desolvation temperature was 450°C. Data were processed using MassLynx software (Waters Corp, 2018).

5.2.4. Microbial community analysis

5.2.4.1. Sample preparation and DNA extraction

For microbial community analysis, raw, washed/thawed and cooked fish were collected. Approximately 10–15 g of the edible parts of the fish were homogenized for one min in 1:1 (w/v) of saline-TE (STE) containing 500 mM sodium chloride (Merck, USA), 50 mM EDTA (Sigma-Aldrich, USA), 50mM Tris (Sigma-Aldrich, USA), at pH 8. Fish homogenates were subjected to different levels of centrifugation (Cambon-Bonavita et al., 2001; Rudi et al., 2004; Svanevik & Lunestad, 2011), starting at 3,000 x *g* for 2 min and 5000 x *g* for 2 min to separate microbial cells from the fish tissue. The supernatants were collected and subjected to higher centrifugation speed of 10,000 x *g* for 5 min to obtain the bacterial pellet used for DNA extraction. Bacterial DNA extraction was continued following the miniprep of genomic DNA method as previously described (Wilson, 1997). In brief, Proteinase K was used to lyse bacterial cells, followed by removal of unwanted material (polysaccharides, proteins, debris) using CTAB. The extracted DNA was finally precipitated using isopropanol. DNA quantification was performed using a Nano Drop 8000 (Thermo Scientific, USA).

5.2.4.2. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The structure of the microbial community of *pindang* from different processing steps was investigated using ARISA. Primers 1406F (5'-TGY ACA CAC CGC CCG T-3') and 23sR (5'-GGG TTB CCC CAT TCR G-3') (Fisher & Triplett, 1999; Jones et al., 2007) were used. PCR reactions contained 10 pmol of each primer, 250 µg/µl of Bovine Serum Albumine, 12.5 µL of MyTaq™ HS Mix (Bioline, AU), no more than 200 ng of DNA template and water to make up a 25 µl reaction (Kent et al., 2004). The PCR was performed based on conditions previously described by Fisher and Triplett (1999) with the following modifications: initial denaturation at 94°C for 2 min, followed by 35 cycles of amplification at 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min and a final extension of 72°C for 2 min. PCR products

were checked by gel electrophoresis. Duplicate PCR products from each sample were pooled and cleaned up using Ultra Clean PCR clean up kit (MOBIO™, AU) prior to separation.

Three different capillary electrophoresis separations for each sample were performed using the Fragment Analyzer™ Automated CE System (Advanced Analytical Technology, Inc., USA) with the following conditions: 5.0 kV injection voltage for 10 sec and 6.0 kV separation voltage for 45 min. Sample preparation and separation followed the manufacturer's protocol using the DNF-910-33 and DNF-915-33 kit (Advanced Analytical Technology, Inc., USA). Data was analysed using PROSize 2.0 (Advanced Analytical Technology, Inc., USA) and only fragments between 50 – 1,500 bp were taken into consideration.

5.2.4.3. Data and statistical analyses

Computer interpolation of fragment sizes at different gel runs often produce imprecise values (Hewson & Fuhrman, 2006). Therefore, to deal with this fragment size imprecision, DNA fragments were binned into operational taxonomic units (OTU) using the custom R script (R Core Team, 2016) by Ramette (2009) using window Size (WS) values 2, 5 and 10 with Shift value (Sh) 1. Using PRIMER 6 software (PRIMER-E Ltd., UK), the best binned outputs were subject to log (X+1) transformation, then the Bray-Curtis similarity coefficient was calculated (Clarke et al., 2006). Using the Bray-Curtis similarity coefficient, a two dimensional non-metric multi-dimensional scaling (2D nMDS) plot was used to display the similarity between samples, and the single linkage cluster was used to determine the indicate percentage of similarity between samples (Blaud et al., 2015; Clarke et al., 2006; Van der Gucht et al., 2005). The goodness of fit of the nMDS plot was presented as a Kruskal stress value (Clarke & Warwick, 2001).

Two-way analysis of similarity (ANOSIM) was performed to test the null hypothesis, that the similarity between samples within pre-defined groups is the same as the average similarity between samples among groups (Rees et al., 2004). In this study, groups were pre-determined as fish sources (local and import fish) and processing steps (raw, washed and cooked fish). The ANOSIM R value

usually ranges between 0-1, where an R value of 0 indicates the null hypothesis is true, and so higher R values show separation between groups (Clarke & Warwick, 2001).

5.2.5. Fish bone analysis and CCPs determination of potential histamine formation during the processing of *pindang*

Fish bone analysis, a type of cause and effect diagram, was used to systematically investigate problems and any potential factors that cause the problems in a food production system (Arvanitoyannis et al., 2009; Charteris, 1993; Ishikawa, 1976; Luo et al., 2018). In this study, this approach was used to identify the causes of histamine formation from each processing steps of *pindang* and to analyse factors that create or contribute the problem.

CCPs from the *pindang* processing were determined based on whether control can be applied in a step and its application is essential to prevent, reduce or eliminate hazard to acceptable levels (FAO & WHO, 1999; FAO & WHO, 2009a) . A decision tree and literature search were used to identify the CCPs. Control and preventive measures were also identified based on the current processing conditions of *pindang* in Sukabumi District, West Java, Indonesia.

5.3. Results

5.3.1. Field observation

Two *pindang* processors used Skipjack tuna (*Katsuwonus pelamis*) while three processors used Eastern Little tuna (*Euthynnus* sp.) as raw materials. Skipjack tuna were caught on a one-day fishing trip in local waters and were processed while fresh (*pindang* type 1), while the Eastern Little tuna were obtained from fish suppliers in frozen form (*pindang* type 2). The “one-day fishing” trip is used by locals to identify fishing activities which are carried out over an entire day. The Skipjack tuna was usually caught using a seine net or “*payang*” vessels that leave the fishing port between 3 - 6 am and return at night between 4 - 8 pm (Marcille et al., 1984; Mujib et al., 2013; Stequert & Marsac, 1989). The processing flowcharts of *pindang* type 1 and 2 are presented in Figure 5-1. The average

weight of Skipjack tuna was three to four kilogram, while the Eastern Little tuna weighted 300 – 400 g per individual. Whole fish were washed, then salt was added before cooking. Wooden wicker was layered and water was added to the bottom of the container, to avoid direct contact between fish and the container. Fish was then arranged in layers and salt was added between layers and on the top layer. The amount of salt used was 5-6% per kg of fish. The topmost layer of fish was covered with thick plastic or paper. The cooked fish was chilled at room temperature and distributed within one to two days. The main difference between the two processes is the thawing of frozen fish in *pindang* type 2. In addition, *pindang* type 1 was wrapped with paper after cooking, while the paper wrapping of *pindang* type 2 was done prior to cooking.

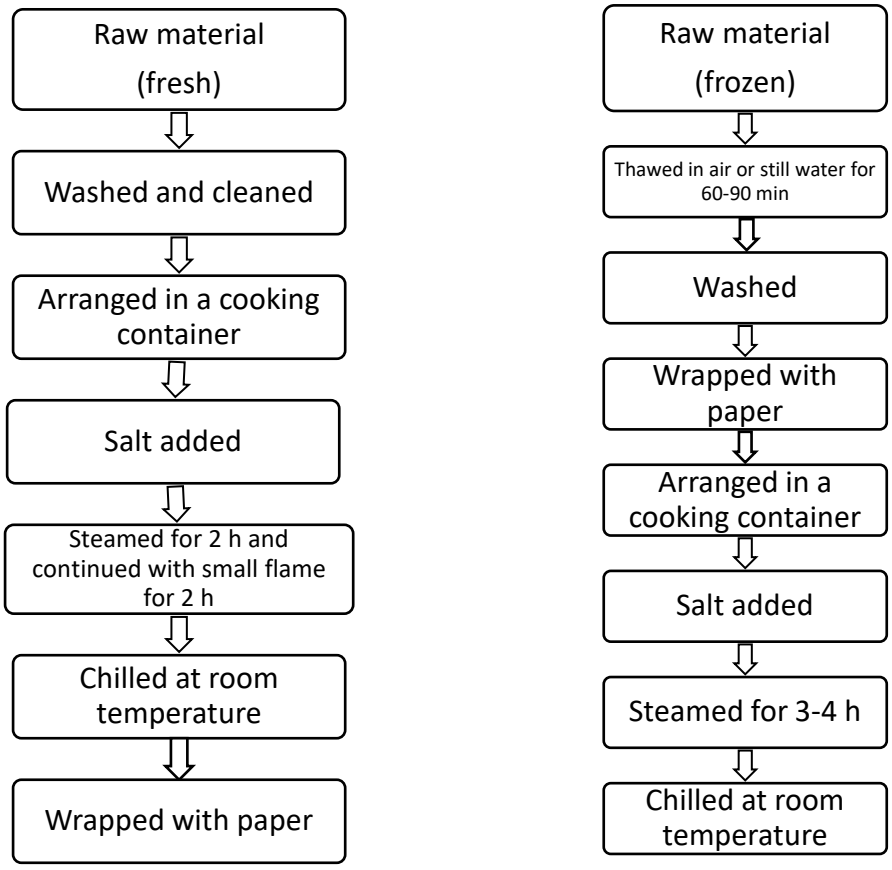

Figure 5-1. Flow diagrams of *pindang* type 1 (A) and type 2 (B) processing

5.3.2. Fish temperature during preparation and cooking

The temperature of raw Skipjack used for *pindang* type 1 was 5-7°C. The preparation step which consisted of washing, arranging and salting was done in less than 30 min, then fish was cooked immediately. The fish temperature before cooking was 17°C. The initial temperature of frozen tuna used for *pindang* type 2 was on average -5°C. The preparation process, which predominantly involves thawing, took 60 to 90 min. The thawing was done in open air and still water (no water replacement during the process). During thawing, the fish temperature increased from -5 to 13-20°C. Two temperature measurements were done during cooking (steaming), *i.e.* at 60 and 90 min. For *pindang* type 1, the average temperature at 60 and 90 min were 79.2±10°C and 83.6±11.5°C, respectively. While for *pindang* type 2, the respective temperature were 99±2.6°C and 93.3±7.6°C.

5.3.3. Microbiological count and histamine content

For both processing methods, the total viable count (TVC) of raw fish varied from 3.0 to 4.5 log CFU/g, while most of the cooked fish has TVC of less than 2 log CFU/g, except cooked fish from processor 2 had TVC of 4.1–5.1 log CFU/g (Table 5-1).

Table 5-1 Total viable counts of raw and cooked fish

Fish processors	Processing type	TVC (Log CFU/g)					
		Raw fish			Cooked fish		
		1	2	3	1	2	3
Processors 1	Type 1	3.3	4.1	3.7	-	2.1	1
Processors 2	Type 1	4.3	3.3	3.5	4.1	4.6	5.1
Processors 3	Type 2	4.1	3.5	3.5	1.3	1.0	1.5
Processors 4	Type 2	4.5	3.3	3.0	-	1.3	2.0
Processors 5	Type 2	4.3	3.3	3.9	2	2	2

Histamine levels of raw and cooked fish collected from five processors are presented in Table 5-2. The histamine levels of *pindang* type 1 were generally lower than type 2. For *pindang* type 2, increasing levels of histamine from raw to cooked fish was observed from processor 3 and 4.

Table 5-2. Histamine levels of raw and cooked fish

Fish processors	Processing type	Histamine level (µg/g)					
		Raw fish			Cooked fish		
		1	2	3	1	2	3
Processor 1	Type 1	1.2	2	0.9	7	1.7	2.3
Processor 2	Type 1	0.8	0.9	0.8	5.8	38	-
Processor 3	Type 2	24	67	173	406	92	277
Processor 4	Type 2	20	12	3.6	141	239	-
Processor 5	Type 2	57	471	437	23	26	12

Note: Each value represents a sample from a different fish

5.3.4. Microbial community of *pindang*

Changes in the microbial composition of *pindang* during processing were examined based on different profiles of the intergenic spacer (ITS) regions from total bacterial DNA of the fish. Using ARISA, 525 fragments between 35 to 1,500 bp were obtained. Binning the DNA fragments (50-1,500 bp) with 2, 5 and 10 window size 1 produced 298, 187 and 116 OTU respectively whilst the correlation coefficient between samples were 0.06, 0.16 and 0.29 for window size 2, 5 and 10, respectively. Binning results from window size 5 were used for further analysis as a compromise between number of OTUs and the correlation coefficient.

The similarity between samples is presented in the 2D nMDS plot (Figure 5-2). The stress value of the MDS plot was 0.18 which is acceptable but suggests that care should be taken in the interpretation of the results (Clarke & Warwick, 2001). The results from cluster analysis showed that at a similarity level of 40%, cooked fish microbiota was separated from most of the raw and washed fish microbiomes, regardless of the fish source. At a higher similarity level of 45%, raw and washed-local fish microbiota was separated from raw and washed-imported fish microbiomes.

Global R values obtained from two way crossed ANOSIM between fish source and between processing steps were 0.314 and 0.321 with P-value 0.003 and 0.001, respectively, showing the overall difference between fish source and processing step are significantly greater than difference between samples within these groups. Further pairwise tests of the processing step group showed significant differences between cooked and raw fish (R=0.609, P=0.003) and between cooked and washed fish (R=0.458, P=0.002), but not between raw and washed fish (R=-0.077, P=0.764). These values supported the MDS observation where the difference in microbiomes between cooked and raw/washed fish was the greatest.

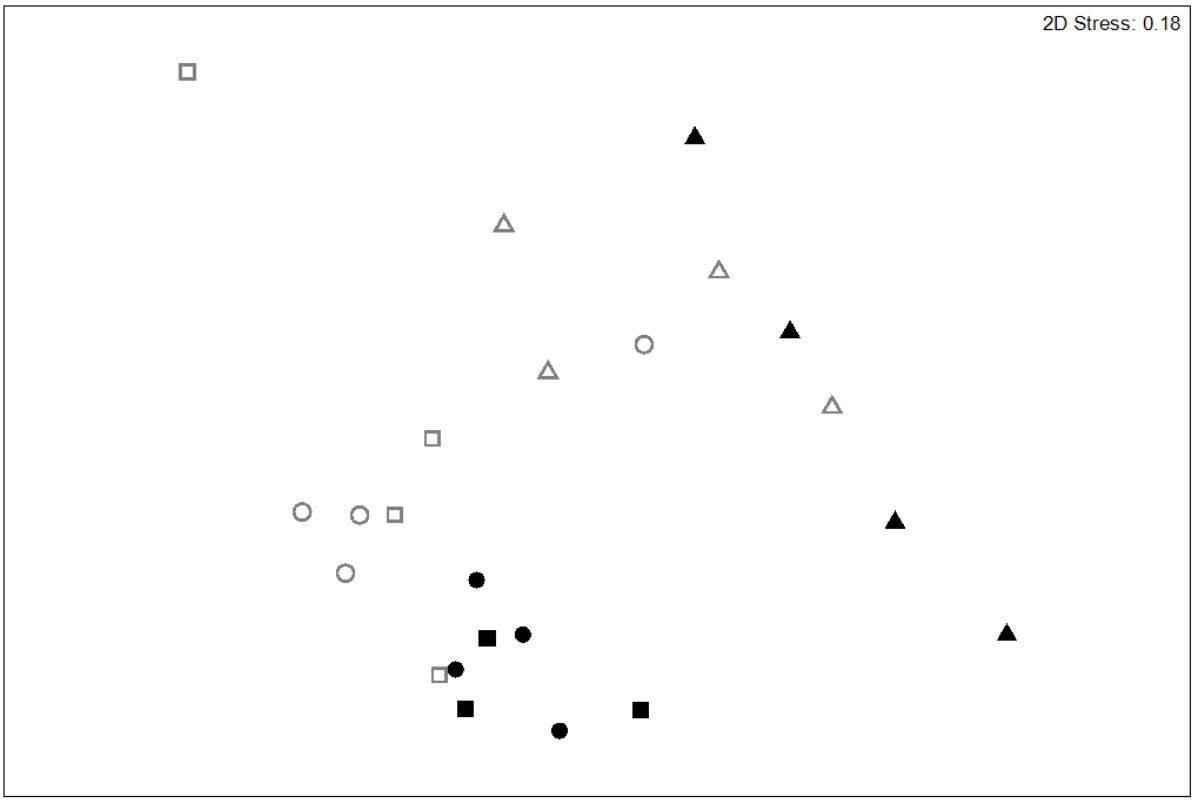

Figure 5-2. nMDS plot of bacterial community profiles of *pindang* generated from distance matrix (Bray-Curtis coefficient) which display similarity between samples.
Note: Samples are represented by the following symbols: Raw fish (circle), washed fish (squares), cooked fish (triangles). Local fish is represented by solid symbols, imported fish is represented by open symbols.

5.3.5. Identified causes of histamine formation and CCPs during the processing of *pindang*

Raw and cooked *pindang* produced from processing type 1 have very low levels of histamine (<50 µg/g), while some of the raw fish used in the processing of *pindang* type 2 had high levels of histamine (>100 µg/g) and these levels increased after cooking. Based on this observation, the processing of *pindang* type 2 was chosen for fish-bone analysis to determine the causes of histamine formation during processing. The histamine levels in the final product depend on the initial level of histamine in raw materials and the activity of HPB during processing. Four main sources (causes) of HPB and histamine during the processing of *pindang* were identified, *i.e.* receiving raw materials, preparation, cooking and post-process handling (Figure 5-3). The preparation steps consisted of fish thawing, washing, wrapping with paper, arranging in cooking container and salting. Inadequate cooling facilities was identified from each step as the main factor that can promote histamine formation by the HPB.

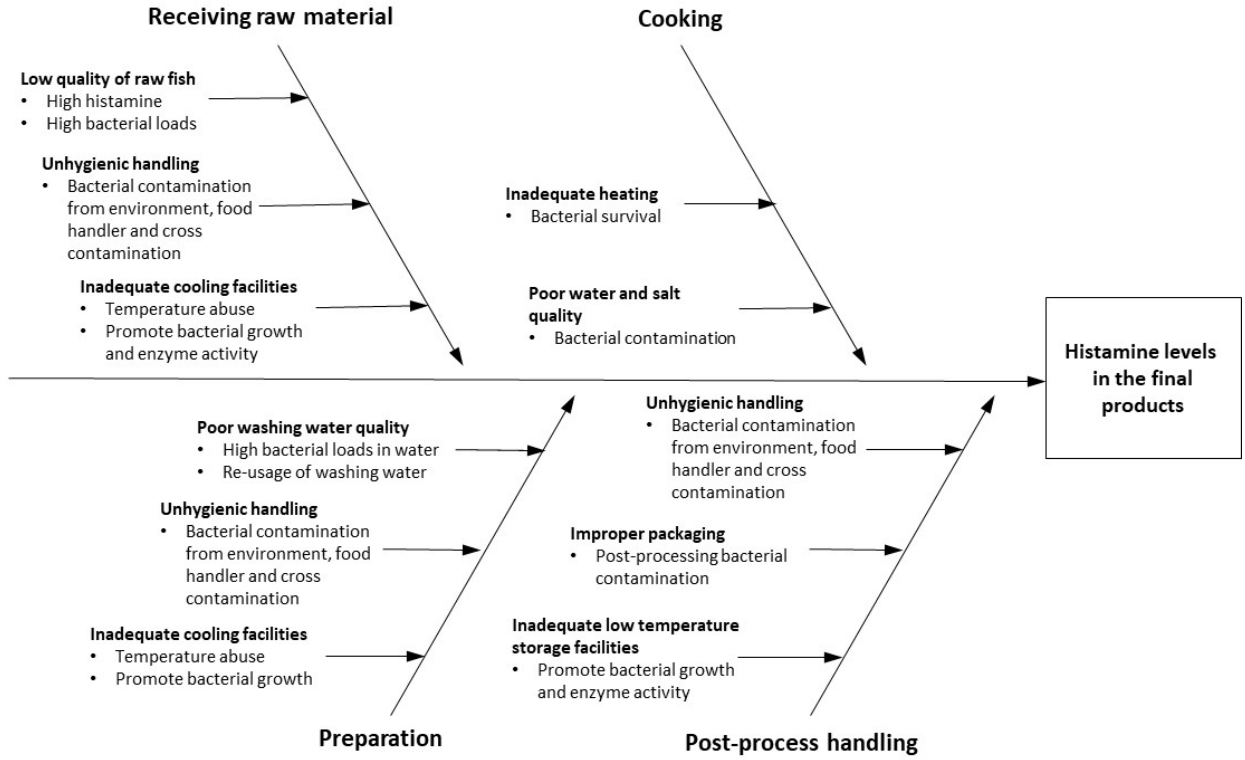

Figure 5-3. Fish-bone analysis of hazards during the processing of *pindang* type 2

Using a decision tree as suggested by the US FDA (US FDA, 2011), four questions were asked to identify the CCP of *pindang* processing (Table 5-3). Six CCPs were identified from *pindang* type 2 processing *i.e*. receiving raw materials, thawing, washing, salting, cooking, and post-process handling. Control and preventive measures for each CCP are presented in Table 5-4.

Table 5-3. Decision tree questions to identified CCPs of *pindang* processing

Process	**Q1: Do preventive control exist?**	**Q2: Is the step specifically designed to eliminate or reduce the likely occurrence of a hazard to an acceptable level?**	**Q3: Could contamination with identified hazard(s) occur in excess of acceptable level(s) or could these increase to unacceptable levels?**	**Q4: Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to an acceptable level?**	**Is this step a CCP?**
Receiving raw material	Y	N	Y	N	Y
Thawing in air or still water for 60-90 min	Y	N	Y	N	Y
Washing	Y	N	Y	N	Y
Wrapping with paper	Y	N	N		N
Arranging fish in the cooking container	Y	N	N		N
Adding salt	Y	Y			Y
Steaming for 3-4 h	Y	Y			Y
Chilling at room temperature	Y	N	Y	N	Y

Table 5-4. CCPs and control measures to prevent histamine formation in *pindang*

Processing step	Hazards	Preventive or control measures	CCPs
Receiving raw materials	- Presence of histamine in raw fish - Presence of HPB in raw fish	- Use fresh (good quality) fish - Keep fish temperature low (5°C)	CCP 1
Thawing	- Growth of HPB as fish exposed to high temperature for long time - Formation of histamine by HPB - Bacterial contamination from water used to thaw the fish - Bacterial cross contamination between fish	- Keep fish temperature low (5°C) during thawing - Use clean and circulated water to thaw fish or alternatively thaw fish in still or moving clean air - Separate thawed fish from frozen one	CCP 2
Washing	- Bacterial contamination from water used to wash fish - Bacterial contamination from fish viscera - Growth of HPB due to time delay	- Use clean water to wash fish - Clean/gut fish properly - Perform this step immediately and avoid time delay	CCP 3
Salting	- Survival of halotolerant HPB	- Add salt as recommended in SNI 2717.3:2009 (10-20% w/w)	CCP 4
Cooking	- Survival of HPB due to inadequate heating - Presence of pre-formed histamine due to its heat-stability	- Heat thoroughly - Use clean water and container to cook	CCP 5
Post-process handling	- Contamination of bacteria from environment due to improper packaging	- Use proper packaging - Cool and store fish in a clean place	CCP 6

5.4. Discussion

According to the processing steps of *pindang* from Pelabuhan Ratu, the product can be categorised as salted *pindang* (SNI 2717.3:2009) . The TVC of raw material and cooked *pindang* should not exceed 5.0 x 10^5 CFU/g, while the maximum allowable histamine level is 100 mg/kg (SNI 2729-2013, SNI 2717.1: 2009) . All raw (fresh and frozen) fish tested in this study had TVC lower than 5.0 x 10^5 CFU/g. Although TVC is not a good hygienic quality indicator of frozen fish, the value is still useful to determine the condition of raw materials (Huss, 1994). TVC of cooked *pindang* from four out of five processors were also very low (less than 2 log CFU/g), and the bacteria were below detection limits in some sample. These TVC values indicated the absence of post-cooking contamination in most *pindang* processing that were investigated. However, higher TVC values observed from cooked *pindang* from processor 2 were possibly due to unhygienic handling when wrapping fish with paper (before storage).

Histamine accumulation depends on the availability of free-histidine as well as the presence of HPB that utilise the Hdc enzyme to convert histidine into histamine (Kim et al., 2002; Lehane & Olley, 2000; Yoshinaga & Frank, 1982). Therefore, fish with a high level of free histidine in their muscle pose a higher risk of histamine accumulation than other types of fish. The histidine of fresh Skipjack tuna was found to be 1,200 – 1,340 mg/100 g (Silva et al., 1998; Suyama & Yoshizawa, 1973), while Little tuna has histidine levels of 1,090 mg/100 g (Suyama & Yoshizawa, 1973). Immediate cooling after harvest is recommended for these fish, especially for fish caught in tropical countries with warm temperature and high humidity, to prevent bacterial growth and histamine formation (Putro & Saleh, 1985; US FDA, 2011). When fish has been exposed to temperatures at more than 83°F (28.3°C), the US FDA suggests immediate chilling on ice, refrigerated seawater, ice slurry or brine (not more than 6 h after fish dead) at 40°F (4.4°C), to prevent rapid formation of the Hdc enzyme (US FDA, 2011).

When fresh Skipjack tuna was used as raw material of *pindang* type 1, the histamine concentrations of raw and cooked fish were very low and no sample exceeded 100 µg/g of histamine. A thawing step is not required, therefore the fish can be processed directly to avoid time delay and abusive temperatures. Another study on *pindang* from Skipjack tuna in Pelabuhan Ratu also showed low levels (2.03 – 26.89 mg/kg) of histamine in the raw and cooked product (Abdillah et al., 2015).

However, when frozen Eastern Little tuna was used as raw material for *pindang* type 2, some raw and cooked fish had histamine levels above 100 µg/g. The frozen fish was purchased from a local fish supplier and there was no available information on how the fish was handled before purchasing. The fish might have come from different fishing vessels, introducing variations on the fish quality including the histamine levels. Fish thawing during *pindang* processing could be the most critical phase for histamine formation since this step was done under uncontrolled temperature. Frozen fish was initially thawed in open air, then the thawing was continued using water in a bucket without recirculation. Although thawing in still or moving air is recommended, the temperature should be maintained at ≤18°C (Jason, 1974). Furthermore, thawing in water should be done using clean and circulated water with temperature to be maintained at ≤20°C (Jason, 1974). These requirements are challenging for the *pindang* processors since most of them are located in rural areas with limited access to cooling facilities (Nurhayati & Purnomo, 2018) and clean water supplies. Bacterial cross contamination between fish and the environment might also occur during this preparation step. Although it only took 60 to 90 min, this step provides favourable conditions for bacteria to grow and might allow any HPB present to produce high levels of histamine. Once formed, histamine cannot be eliminated by heating or cooking (Kim et al., 2002; US FDA, 2011), and can still be detected in the cooked fish.

The amount of salt added was 6% per kg of fish and salting was done shortly before cooking. Therefore, salting might not be sufficient to prevent the growth of any HPB which may take place

during the preparation phase. Several studies suggested the presence of halotolerant HPB such as *Vibrio, Staphylococcus* and *Pseudomonas* might contribute to the formation of histamine in the fish product (Yatsunami & Echigo, 1992). These species can grow and produce histamine at 10-15% NaCl in histidine broth or 12% NaCl in sardine (Yatsunami & Echigo, 1992, 1993). For salted fish products, a combination of salt and refrigeration was more effective to prevent histamine formation (Lehane & Olley, 2000). Very low levels of histamine (<0.5 mg/kg) were observed in refrigerated brined-anchovies (14% salt w/v), while at room temperature the histamine levels of the anchovies reached >500 mg/kg, inversely related with the salt concentration (Karaçam et al., 2002). This approach might be suitable for application to cook *pindang*. High salt in the final product could prevent the growth of bacteria which came from post-processing contamination. Further storage at refrigerated temperature could therefore improve the product shelf-life.

ARISA was able to detect differences in the microbial community profiles of the *pindang*. The bacterial composition of fish species is affected by a combination of several factors, including both pre-harvest (Gatesoupe et al., 2013; Giatsis et al., 2015; Larsen et al., 2014; Wilson et al., 2008) and post-harvest treatment and handling (Ge et al., 2012; Svanevik & Lunestad, 2011). *Pindang* uses different species of tuna caught from different waters as raw material, thus the observed microbial community patterns were different. Furthermore, each type of fish experienced different handling and post-processing treatment which probably influenced their bacterial community profiles.

The microbial communities from the raw and washed fish were not significantly different, which is probably due to the dominance of bacteria specific for tuna species in both samples. Fish microbial communities have been suggested to comprise two populations: the core and transient bacterial population (Chaillou et al., 2015; Wilson et al., 2008). The core population remains in the fish as part of their intestinal microbiota, while the transient population changes based on the environment. In contrast, the finished or cooked product has significantly different microbial communities compared to the raw and washed fish. Apart from the effect of salt addition and

heating on bacterial elimination, post-processing contamination might influence the formation of different bacterial community profiles of cooked fish.

ARISA is fast, reliable and relatively cheap method of microbial community fingerprinting. It is also relatively cheaper compared to the 'next generation' sequencing, but with comparable output in terms of its ability to capture significant community changes due to temporal, seasonal and treatment shift (Jami et al., 2014; van Dorst et al., 2014). This technique can be used to determine whether new intervention strategies in fish processing affects the overall microbial community of the products, although it cannot be used to evaluate quantitatively the efficacy of such intervention. Another limitation of this technique is its inability to identify the dominant taxa or species within the community. Also, it tends to underestimate the species richness, because one single peak or fragment produced from ARISA may consist of more than one microorganism. Thus further analysis is important to identify taxa of specific interest (Brown et al., 2005; Kovacs et al., 2010).

HACCP is a useful approach to improve the food production process and enhance food product safety and quality(FAO & WHO, 1999; FAO & WHO, 2009a). This study focused on the likelihood of histamine formation during *pindang* processing, therefore the presence of HPB and histamine in the processing steps were identified as hazards. Previous studies have identified at least three critical control points (CCPs) of *pindang* processing in Indonesia, *i.e.* materials receiving, cooking, and post-processing handling (Purba, 2010; Thaheer et al., 2010). The identification of these CCPs was not only based on the presence of HPB and histamine but also other types of hazards such as pathogenic bacteria, formaldehyde, heavy metals and foreign materials. Apart from these CCPs, three additional CCPs were determined from the current processing of *pindang* type 2, *i.e.* thawing, washing, and salting. The use of fresh fish is recommended as the first control or preventive measure as the levels of histamine in raw fish will determine the levels of histamine in the final products. Since histamine is a heat-stable amine, pre-formed histamine cannot be eliminated by subsequent processing step (heating) (US FDA, 2011; Visciano et al., 2014). Apart from that,

maintaining appropriate fish temperature throughout the processing is an essential measure to control the growth and activity of HPB.

Information on the CCPs during *pindang* processing is useful to assist the processor to improve their processing practice. The processors should be able to implement the suggested control or preventive measures of CCPs to avoid histamine formation and/or accumulation in the final product, even though the HACCP system is not yet established in their processing facilities. Several prerequisite programmes such as Good Hygienic Practices (GHP), Codex Codes of Practice and other food safety requirements as determined by the competent authorities should be in place before the processors can apply the HACCP procedures in their processing facilities. Hygiene and sanitation practices of *pindang* processing in Indonesia is specifically regulated in SNI 2717.3-2009. However, this regulation is not only for tuna-based *pindang* but also for *pindang* made from other non-Scombroid fish. Therefore, it does not explicitly mention HPB and histamine as hazards in the *pindang* processing. Furthermore, this regulation aims to control hazards based on the general steps of *pindang* processing, such as receiving raw materials, gutting, washing, salting, cooking, cooling, packaging and storing. Since the used of frozen fish is not popular amongst non-Scombroid *pindang*, the thawing step is not included as a critical step in *pindang* processing in this regulation.

As suggested by Buchanan (1993), integrated and dynamic predictive models in HACCP system can be used to quantify the effect of each intervention strategy applied in each step of food production, and to estimate the contribution of these strategies to achieve the defined safety criteria (safety critical limits). For example, when information related to a microorganism of concern is available from published literature, a quantitative risk assessment can be used to predict the behaviour of the microorganism towards different processing strategies as determined in the HACCP plan. Based on these behaviours, it can be decided whether that microorganism is a potential hazard or not, and if so, how the control measures should be applied to reduce or to eliminate the hazard caused by the pathogen (Notermans et al., 1995). For *pindang* processing in Indonesia, predictive modelling of HPB growth and histamine formation, as presented in Chapter 3 and 4, can be used as

input information for HACCP planning to evaluate the effectiveness of control measures such as low-temperature or salt addition, to reduce or to eliminate histamine formation and thus prevent HFP cases due to *pindang* consumption.

5.5. Conclusion

Two types of *pindang* processing were studied in Pelabuhan Ratu, Sukabumi District, West Java Province, Indonesia. The levels of histamine in *pindang* from Indonesia were found to depend on the quality of raw materials and the processing practices. To ensure the safety of *pindang* produced by traditional fish processors in Indonesia, the use of fresh fish as the raw material is suggested. Alternatively, frozen fish might be used but care should be taken during the preparation step including maintaining low fish temperature during thawing and avoiding time delays. The identified CPPs and control/preventive measures can be used as a guideline to assist processors to improve their current processing practice.

Chapter 6. General Discussion, Conclusions and Recommendations

Pindang is a potential source of animal protein for Indonesian's that is relatively cheap, easy to obtain as it's available in most traditional markets, tasty and can be cooked into a variety of dishes. As a typical traditional food, *pindang* industries are owned by a family or a group of local people. One of the major issues of *pindang* is the product safety related to histamine contamination. There are several reports of HFP cases in Indonesia indicating *pindang* as the main cause of histamine illness (Ainun, 2015; Anonymous, 2004, 2008, 2014, 2016). *Pindang* is generally prepared from tuna, and this group of Scombroidae fish has naturally high levels of histidine in their muscle (US FDA, 2005). Several factors can trigger the conversion of histidine into histamine in fish, including the presence of HPB that can activate the histidine decarboxylase (Hdc) enzyme to catabolize histidine into histamine.

The main purposes of this thesis were to understand the behaviour of HPB in conditions that mimic the processing of *pindang* in Indonesia and to assess the risk of histamine formation during processing. The outcomes from this thesis were used to propose intervention strategies or recommendations that will improve the processing and maintain the safety and quality of *pindang*. Baseline information about processing conditions as well as fish samples were collected from Sukabumi District, West Java Province, as one of the biggest *pindang* processing centres in Indonesia (Heruwati, 2002).

Since 2012, the availability of fresh tuna caught from local waters in Indonesia has been limited and frozen tuna is being used as an alternative raw material in *pindang* processing. This situation leads to the introduction of a thawing step during processing, which also extends the processing time. Thawing procedures for quick-frozen fish blocks were set in the Codex STAN 165-1989. When fish is thawed in air, the air temperature should not exceed 25°C, while the water immersion thawing should use a circulating water system with temperature to be maintained at 21°C (FAO & WHO, 1989). However, this condition is barely achieved in most *pindang* processing

units that are located in the coastal areas of Indonesia (MMAF RI, 2017; Nurhayati & Purnomo, 2018), which may cause significant problems of histamine intoxication. Furthermore, although HPBs comprise only a small amount of the total natural microflora in live fish and are mostly found in gills, skin or gastrointestinal tract (FAO & WHO, 2012; Lehane & Olley, 2000), controlling the growth of these bacteria by maintaining temperature during processing is essential.

6.1. Summary of findings

As shown in Chapter 2 of this thesis, *pindang* collected from fish processors in Sukabumi and made from frozen raw materials were susceptible to HPB contamination. Different genera of high histamine producers were found from *pindang* made from frozen tuna, including *Enterobacter* sp., *Klebsiella* sp., *M. morganii* and *Providencia* sp. Further confirmation showed that one of the isolates (*E. aerogenes*) could produce more than 4,000 μg/ml of histamine in histidine broth after incubation at 30°C for 24 h. However, when fresh tuna was used as raw material, no high histamine producers were found in the samples. This observation indicated that the current thawing process for frozen raw tuna might have significant effects in supporting the growth of pre-existing HPB and promoting HPB contamination.

A screening method to identify the presence of HPB in a fish product is important to take actions to prevent the activation of Hdc enzyme and further formation of histamine in fish. Identification of HPBs in fish can be done using conventional and molecular approaches. Most of the conventional methods use differential media to assist the visualisation of the HPB colonies. The media are enriched with histidine as a source of amino acid and included a pH indicator. Bacterial decarboxylation of histidine into histamine will increase the pH of the media and colour changes will be observed (Kim et al., 2001; Niven et al., 1981; Shelef et al., 1998; Yamani & Untermann, 1985). Moreover, molecular techniques have been developed to identify specific HPB. Different primers targeting the *hdc* gene that is responsible for Hdc enzyme production in Gram-negative HPB have been suggested (de Las Rivas et al., 2005; Kanki et al., 2002; Kim et al., 2003a; Takahashi et al.,

2003). This method using PCR requires less time and provides more specific results than the conventional cultural method.

E. aerogenes isolated from *pindang* was used in the studies described in chapters of this thesis because the isolate produced a high level of histamine and, thus, represented a high histamine producer. Mathematical modelling, which enables the prediction of growth and histamine formation of *E. aerogenes* under conditions (temperature and salt concentrations) that mimic the processing of *pindang*, was presented in Chapter 3. The isolate was grown at 10, 15, 20 and 30°C with no salt, or 6, 10 and 20% NaCl in the media. Within the range of temperature used in this study, the optimum growth of *E. aerogenes* was at 30°C, without additional salt in the media. The estimated minimum growth temperature (T_{min}) of *E. aerogenes* in this study was 3.9°C, higher than the T_{min} of other HPB such as *M. morganii* (Emborg & Dalgaard, 2008a), *H. alvei, S. liquefaciens* and *E. agglomerans* (Ridell & Korkeala, 1997) that are reported to range from 0.3 – 2.8°C. Further findings showed the maximum growth rate (μ_{max}) of *E. aerogenes* in this study was affected by a combination of temperature and salt concentration. Interestingly, another study of *E. faecalis* showed that the isolate was more susceptible to high salt concentration than temperature (Gardini et al., 2001).

A toxic level of histamine was formed after 8 h of incubation at 30°C without added salt, and the highest concentration of histamine was produced at this condition. At the same temperature, the isolate was able to grow at 6% NaCl and produced >300 µg/ml of histamine after 33 h of incubation. At higher salt concentration (>10%), histamine was not formed, although to get an estimated time to reach 100 µg/ml at 30°C, 10% NaCl was included in the model development. Therefore, a combination of low incubation temperature and high NaCl was effective to prevent the growth and histamine formation of *E. aerogenes* in broth. Salt has been shown to have an inhibitory effect against the growth and histamine formation of other HPBs, such as *K. pneumonia* (Taylor & Woychik, 1982), *E. faecalis* (Gardini et al., 2001), *M. psychrotolerans* (Emborg & Dalgaard, 2008b). However, a lower concentration of salt (less than 3%) had insufficient inhibitory effects on

Enterobacter spp. growth and, thus, was less effective at preventing histamine accumulation (Greif et al., 2006). In this study, the effects of salt addition on bacterial growth and histamine formation were complementary, showing that the salt inhibited the activity of Hdc in bacterial cells thus decreasing the likelihood of histamine formation (Sumner et al., 1990).

In Chapter 4, a validation experiment was done using Grey mackerel (*Scomberomorus semifasciatus)*. The current practice of *pindang* uses 6% NaCl (w/w), while SNI 2717.3:2009 suggests using 10 – 20% of salt per kg fish. Results from the experiment in this chapter showed a similar trend to the broth experiment (Chapter 3). However, for the no salt treatment, the histamine levels in fish were higher than in the broth and with 6% NaCl, the histamine in fish were significantly higher than in the broth. The addition of 6% NaCl significantly lowered the amount of histamine produced by *E. aerogenes* in fish, although toxic concentrations of histamine (>100 µg/g) were still found at the end of storage periods, which were 35 h and 84 h for incubation at 30 and 20°C, respectively. The ability of salt to prevent histamine formation has also been observed in different types of foods, such as brined anchovies (Karaçam et al., 2002) and during miso fermentation (Chin & Koehler, 1986).

Under some incubation conditions, *E. aerogenes* in fish was able to produce higher levels of histamine than in broth. However, the yield of histamine produced per cell of *E. aerogenes* in fish was lower than in broth. This yield factor is used to correlate the absolute rate of *E. aerogenes* with the rate of histamine production, since the numbers of microorganisms determine the levels of histamine formed (Rodriguez-Jerez et al., 1994). As the bacterial growth rate in fish was also lower, the overall rate of histamine formation by *E. aerogenes* in fish was slower than in broth and the time to reach a toxic level (*i.e.* 100 µg/ml) was longer. However, the histamine yield model performed better to predict the levels of histamine in fish than in broth. The estimated NaCl concentration and temperature that prevent the formation of 100 µg/ml of histamine were 13.2% and 6.1°C, respectively. In the model (Equation 3-11), these values were obtained by combining data from observed and estimated times to reach 100 µg/ml histamine which, in the absence of measured histamine levels, were deemed to equal to 7 log CFU/ml of *E. aerogenes*.

Predictive modelling can be used as a tool to estimate the growth and histamine formation of HPB that potentially contaminate *pindang* during processing, based on temperature and salt concentration used in the processing.

From the results presented in Chapter 4, it can be suggested that the use of higher concentrations of salt are more effective to prevent histamine formation in *pindang*. However, based on information collected from the fish processors, the price of salt in the local markets fluctuates and sometimes is unreasonably high. In this situation, lowering the amount of salt added to the fish can reduce the production costs. Consequently, in these situations low temperature during processing should be maintained thoroughly in each processing line.

Field observations of *pindang* processing in Sukabumi were presented in Chapter 5 of this thesis. In general, the processing flow has followed the guideline set in SNI 2717.3:2009, except that the processors use less salt than recommended and no fish gutting is done prior to cooking. The processors also use paper to wrap each individual fish before arranging the fish in a cooking container. *Pindang* made from frozen tuna have higher histamine levels than those prepared from fresh tuna. The initial levels of histamine in raw frozen tuna varied from 3.6 to 437 µg/g and in the cooked *pindang* the levels varied from 12 to 406 µg/g. Histamine formation probably occurs during fish preparation and accumulates in the final product. Investigation of *pindang* made from fresh tuna showed lower levels of histamine contamination (0.8 – 1.2 µg/g in fresh tuna and 1.7 – 38 µg/g in cooked *pindang*). These findings were in accordance with findings from Chapter 2 which showed that *pindang* made from frozen tuna was more likely to be contaminated with HPB than *pindang* from fresh tuna, thus potentially having a higher level of histamine.

Commercially, the use of frozen tuna as a raw material is preferred over fresh tuna because the frozen fish are relatively cheap and available all year-round, while the supply of fresh tuna depends on the capture from local fisherman. However, a thawing step is introduced to *pindang* processing when frozen tuna is used as the raw material. Thawing was categorized as part of the

preparation steps in *pindang* processing where histamine contamination might occur. Therefore, thawing was identified as a CCP for histamine formation in *pindang* processing, together with the other steps, *i.e.* receiving materials, washing, salting, cooking and post-process handling. Visual observations and fish temperature records that were made in two different *pindang* processing units in Sukabumi confirmed that temperature control during *pindang* processing was inadequate, especially in the preparation step which includes thawing.

Further investigation on how *pindang* processing practices (freezing, thawing and cooking) affect the growth of HPB and the histamine formation in *pindang* was presented in Chapter 3 and 4 of this thesis. Several parameters in the experiments undertaken were designed based on the latest observation of *pindang* processing in Sukabumi (Chapter 5).

Based on these observations, an experiment in broth (Chapter 3) was done with different initial levels of *E. aerogenes* and histamine concentrations. Low initial counts and histamine concentration represent fresh raw materials, while high initial counts and histamine concentration represent pre-contaminated raw materials. Temperatures used in the thawing experiment represented the current thawing temperature (25°C), the recommended air and water thawing temperatures (18°C) (Jason, 1974), as well as the common temperature of refrigerated thawing (4°C).

The final concentration of histamine in broth culture depended on the initial density of *E. aerogenes* and initial histamine levels. *E. aerogenes* was susceptible to freezing and cooking temperature, especially at low initial density. Although the number of the *E. aerogenes* remained similar during 4 h thawing at 4, 18, 25°C, some of the cells' biochemical reactions recovered rapidly. This was markedly shown by the group with high initial contamination. More than two-fold increases in histamine concentration were observed at the end of thawing period. As suggested, the pre-formed Hdc enzyme might remain active after freezing, thus the histamine production was still observed despite the absence of HPB (Fujii et al., 1994; ICMSF, 2005; Takahashi et al., 2003).

To validate the result from the broth experiment, another experiment using Blue mackerel (*Scomber australasicus*) was presented in Chapter 4. Artificial contamination with *E. aerogenes* culture before freezing was done to emulate the potential histamine formation in fish prior to the processing. The experimental design of Chapter 4 was based on a worst-case scenario, where frozen pre-contaminated fish was used as raw material in *pindang* processing then thawed at 25°C for 4 h. The thawing time was two times longer than the normal time required by the fish processors to thaw the frozen fish. To mimic the real processing of *pindang,* 6% NaCl was added to the fish before cooking.

Microbiological observations from this study showed similar trends to the broth experiment (Chapter 3). A slight decrease of *E. aerogenes* counts was observed after fish were frozen. However, at an optimum growth temperature (25°C) (Tsai et al., 2005a), *E. aerogenes* survived freezing and grew to reach similar levels as the initial counts at the end of thawing. Cooking the fish was sufficient to kill the *E. aerogenes*, as no colonies were found in cooked *pindang*.

In contrast, the trends in histamine concentration change during thawing and after cooking were different between the fish and broth experiments. At the same thawing conditions, histamine in broth increased during thawing and decreased after cooking, while in fish the histamine decreased during thawing and remained stable after cooking. The water-solubility of histamine is suggested as the main cause of the decreased concentration observed in fish, as the melting water released from the fish may carry histamine away with it (Hongpattarakere et al., 2016; Shakila et al., 2005). While in broth, water dripping or loss did not occur as the volume of the liquid remained constant during treatment, therefore histamine accumulated in the media. In other studies, boiling was more effective to reduce histamine in fish compared to other cooking methods such as frying and grilling (Chung et al., 2017; Paulsen et al., 2006). This finding is more relevant to broth conditions than the fish experiment in the current study. Furthermore, the proposed mechanism of histamine reduction with boiling was similar to the dilution mechanism of histamine by the melting water during thawing.

Although the histamine reduction in fish could potentially be achieved by thawing or boiling the fish, other results from Chapter 4 showed that high concentrations of histamine were still found in *pindang* following storage at 4 and 25°C. High pre-contamination levels of histamine in fish could not be eliminated with the current cooking practice of *pindang*, therefore raw fish with minimum microbiological and chemical requirements as set in the SNI 2729-2013 should be used to avoid histamine accumulation.

6.2. Conclusions and recommendations

It can be concluded that the microbiological and chemical safety of *pindang* were determined by the quality of raw materials used in the processing, the amount of salt added to the fish and the ability to maintain a low temperature during processing. To support this, the current SNI for *pindang* processing (SNI 2717.3:2009) needs to be reviewed by including thawing conditions in the processing diagram. This is because the use of frozen fish as raw materials for *pindang* has become more common and more feasible for the fish processors. The proposed changes of *pindang* processing flowchart are given in Figure 6-1.

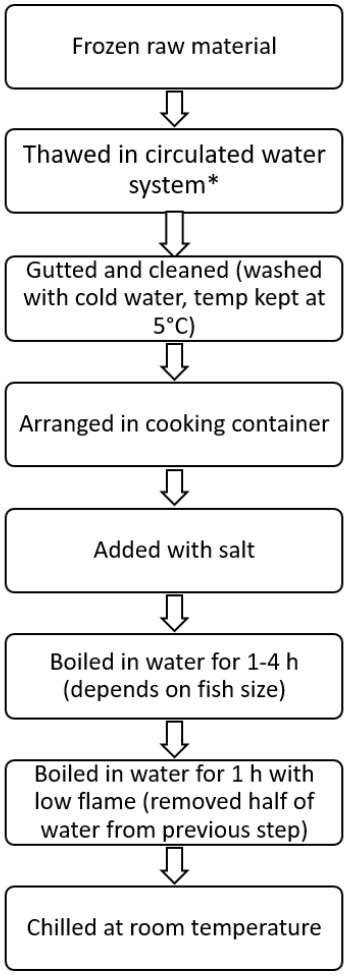

Figure 6-1. The proposed change of *pindang* processing flow (*)

Additional notes:

- Thawing in a circulating water system should be done by maintaining water temperature at less than 18°C.
- Thawing is completed when fish can be easily separated without damaging the fish surface, while the internal temperature of the fish block should not exceed 7°C.

FAO suggested the use of the water-thawing system for whole fish blocks (Jason, 1974). This simple system primarily transfers heat from the water to the surface of frozen fish. The system consists of a tank with several fish block trays or containers and separators (baffles) for water movement (Figure 6-2). A temperature controller is inserted to maintain water temperature not exceeding 18°C. The water inlet and outlet are also attached to the system to regulate water circulation.

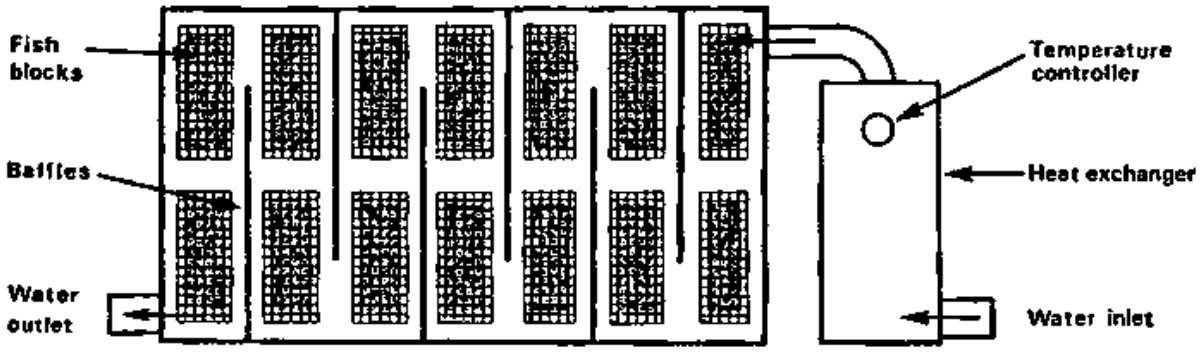

Figure 6-2. Simple immersion thawing system, reproduced from Jason (1974)

Fish thawing in Sukabumi is currently done by using a combination of air and water thawing. However, the time and temperature during thawing are not monitored. The circulated-water thawing system as suggested by the FAO is suitable to replace the current thawing facilities in *pindang* processing units in Sukabumi. This system is relatively cheap and fast compared to other thawing systems such as air blast or still-air that also need a large thawing area and a longer time to complete. The use of a temperature controller that requires electricity might be eliminated when cold water supplies are available. However, continuous supplies of clean and cold water are necessary to avoid accumulation of bacterial loads in the water tank. The local and central competent authorities could also actively support the development of better *pindang* processing by providing this thawing system (equipment), especially for processors who use frozen raw tuna.

Another recommendation regarding the amount of salt to be added during the processing is also suggested. The current recommendation is to use 10 – 20% of salt per kg fish. This recommendation can be replaced with flexible options for different conditions of the processing units. For example, if no cooling facilities were available in the processing plant, a higher salt concentration (10%) should be added. However, a lower salt concentration (6%) might also be used when the processing temperature can be maintained at less than 18°C. This strategy could reduce the cost to the processors due to the increasing salt price.

Intervention strategies and suggestions made from this thesis could assist *pindang* processors to improve their production process and to produce *pindang* with higher levels of safety and quality. The introduction of a new technology should be done gradually, however, especially when the technology will replace the current practices of the community. Different challenges might occur at the early stages of the introduction, such as reluctance from the processors to adopt the new technology, low financial resources, as well as the lack of skills and technology access of the processors. Therefore a pilot project to introduce and apply the circulated thawing system in *pindang* processing is important to ensure a prolong utilization of the equipment. This first step could help to identify potential problems and to make the necessary adjustment of the new system with the current condition of the production plant. In addition, education and training on food-technology related areas, including food safety, should also be continuously delivered to the fish processors to update their knowledge and skills.

A quantitative risk assessment on the prolific HPB is proposed to extend our understanding of the potential risk of HFP from consuming *pindang* and to calculate the real health and socio-economic burden associated with this product. Based on the six identified CCPs, the most important factor to control the hazard is temperature, therefore a comprehensive modelling study that provides quantitative information on the effect of temperature and time at each processing step on the HPB growth and histamine production is important. These models can be used to support the evaluation of control and preventive measures efficiency during the processing and to determine whether such measures need some adjustment to achieve better performance, *i.e*., to develop a risk-based HACCP plan.

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